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Docket No.: PF-0358-2 DIV

Response Under 37 C.F.R. 1.116 - Expedited Procedure
Examining Group 1653

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of: Bandman et al.

Title:	HUMAN RETICULOCALBIN ISOFORMS		
Serial No.:	09/847,809	Filing Date:	May 01, 2001
Examiner:	Carlson, K.	Group Art Unit:	1653

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BRIEF ON APPEAL

Sir:

Further to the Notice of Appeal filed November 6, 2003, and received by the USPTO on November 10, 2003, herewith are three copies of Appellants' Brief on Appeal. Appellants hereby request a 1-month extension of time in order to file this Brief. Authorized fees include the statutory fee of $110.00 for a 1-month extension of time, as well as the $ 330.00 fee for the filing of this Brief.

This is an appeal from the decision of the Examiner finally rejecting claims 10, 30, 31, 33, 36, 37, and 39-42 of the above-identified application.

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(1) REAL PARTY IN INTEREST

The above-identified application is assigned of record to Incyte Pharmaceuticals, Inc. (now Incyte Corporation, formerly known as Incyte Genomics, Inc.) (Reel 8922, Frame 0082), which is the real party in interest herein.

(2) RELATED APPEALS AND INTERFERENCES

Appellants, their legal representative and the assignee are not aware of any related appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the instant appeal.

(3) STATUS OF THE CLAIMS

Claims rejected:	Claims 10, 30, 31, 33, 36, 37, and 39-42
Claims allowed:	None
Claims canceled:	Claims 1-9, 11-28 and 45-48
Claims withdrawn:	Claims 29, 32, 34, 35, 38 and 43-44
Claims on Appeal:	Claims 10, 30, 31, 33, 36, 37, and 39-42 (A copy of the claims on appeal can be found in the attached Appendix).

(4) STATUS OF AMENDMENTS AFTER FINAL

There were no amendments submitted after Final Rejection;

(5) SUMMARY OF THE INVENTION

Appellants' invention is directed, *inter alia,* to isolated antibodies which specifically bind to human reticulocalbin isoform RCNδ, a polypeptide comprising the amino acid sequence of SEQ ID NO:3. Nucleic acids encoding the RCNδ of the present invention were first identified in Incyte Clone 1601793 from the bladder cDNA library (BLADNOT03) using a computer search for amino acid sequence alignments (Specification at page 17, lines 15-17). The amino acid sequence of SEQ ID NO:3, as shown in Figures 2A, 2B, 2C, 2D, 2E, 2F, and 2G [of the Specification]...is 315 amino acids in length, has an N-terminal signal peptide (M-1 to S-19), and has seven EF-hands (Specification at

page 17, lines 22-25). As shown in Figures 4A and 4B [of the Specification], RCNδ has chemical and structural homology with human reticulocalbin (GI 1262329; SEQ ID NO:5). In particular, RCNδ and human reticulocalbin share 58% identity, share an N-terminal signal peptide, and six EF-hands. As illustrated by Figures 5B and 5C [of the Specification], RCNδ and human reticulocalbin have rather similar hydrophobicity plots. Northern analysis shows the expression of this sequence in various libraries, at least 50% of which are immortalized or cancerous, at least 22% of which involve immune response, and at least 19% of which involve fetal or proliferating tissue. Of particular note is the expression of RCNδ in heart, gut, prostate, and smooth muscle; and at sites of hematopoiesis (Specification at page 17, line 29 to page 18, line 7). Overexpression of reticulocalbin mRNA has been associated with the increased matrigel invasive properties of three human breast cancer cell lines. Conversely, reticulocalbin was not found to be expressed in two poorly invasive breast cancer cell lines (Liu, Z. et al. (1997) Biochem. Biophys. Res. Comm. 231:283-289). (Specification at page 3, lines 8-11.) The antibodies, compositions and methods of the present invention are useful for, *inter alia*, disease detection and expression profiling as well as in toxicological testing applications.

(6) ISSUES

1. Whether claims 10, 30, 31, 33, 36, 37 and 39-42 directed to isolated antibodies which specifically bind to SEQ ID NO:3 meet the novelty requirement of 35 U.S.C. §102(a).

2. Whether claims 10, 30, 31, 33, 36, 37 and 39-42 directed to isolated antibodies which specifically bind to SEQ ID NO:3 meet the novelty requirement of 35 U.S.C. §102(b).

3. Whether claims 36 and 39 directed to isolated antibodies which specifically bind to SEQ ID NO:3, produced by the methods of withdrawn claims 35 and 38, meet the definiteness requirement of 35 U.S.C. §112, second paragraph.

(7) GROUPING OF THE CLAIMS

As to Issue 1

All of the claims on appeal are grouped together.

As to Issue 2

All of the claims on appeal are grouped together.

As to Issue 3

Claims 36 and 39 are grouped together.

(8) APPELLANTS' ARGUMENTS

Issue 1--Claim rejections under 35 U.S.C. § 102(a)

Claims 10, 30, 31, 33, 36, 37 and 39-42 have been rejected under 35 U.S.C. § 102(a) as allegedly being anticipated by Yabe et al. (July 18, 1997; J. Biol. Chem. 272:18323-18239; of record, copy supplied for the convenience of the Board as Attachment 1). Appellants respectfully disagree with the Examiner's analysis of the art in Yabe et al. and submit that claims 10, 30, 31, 33, 36, 37 and 39-42 are not anticipated under 35 U.S.C. § 102(a) by this publication.

For a reference to anticipate claimed subject matter under any section of 35 U.S.C. § 102, "every element of the claimed invention must be identically shown in a single reference." *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990). Furthermore, the MPEP provides guidance for interpretation of the case law that has been generated with regard to 35 U.S.C. § 102: "the reference ***must teach every aspect of the claimed invention either explicitly or impliedly***. Any feature not directly taught must be inherently present" M.P.E.P. § 706.02 (emphasis added). Moreover, M.P.E.P. § 2112 states that "[t]he fact that a certain result or characteristic **may** occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic" (emphasis in original). M.P.E.P § 2112 further states that "[t]he examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic **necessarily** flows from the teachings of the applied prior art" (emphasis in original). Yet the Final Office Action provides no such basis or reasoning. Furthermore, the Examiner has attempted to support this rejection with articles by Bost et al., and Bendayan (of record; copies supplied for the convenience of the Board

as Attachments 2 and 3 respectively), Abaza et al. (of record; copy supplied for the convenience of the Board as Attachment 4) and Li et al. (of record; copy supplied for the convenience of the Board as Attachment 5); and with U.S. Pat. No. 6,210,670 (Berg; of record; copy supplied for the convenience of the Board as Attachment 6). Appellants strongly disagree with the Examiner's position for the following reasons.

I. Claims 10, 30, 31, 33, 36, 37 and 39-42 are not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

Claim 10 recites "An isolated antibody which ***specifically binds*** to a polypeptide comprising the amino acid sequence of SEQ ID NO:3." (Emphasis added.) In order to anticipate the claimed subject matter under any section of 35 U.S.C. § 102, "the reference ***must teach every aspect of the claimed invention either explicitly or impliedly***" M.P.E.P. § 706.02 (emphasis added). Under these parameters, the claimed subject matter is not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

It appears that the basis of this rejection is the Examiner's assertion that "Yabe et al....teach calumenin having 98.2% identity to SEQ ID NO:3. On page 18234, col. 1, para. 3, Yabe et al. made antibodies against calumenin, anti-protein-disulfide isomerase antibody" (Final Office Action at page 3). The Examiner then concludes that "[g]iven that antibodies bind epitopic structures rather than sequences per se, and the identity between RCN and calumenin is high, the antibody made by Yabe et al. will also bind polypeptides having SEQ ID NO:3."

First note that Yabe et al. made no antibodies. The four antibodies used in the experiments described by Yabe et al. were all commercially available antibodies made to antigens other than "a polypeptide comprising the amino acid sequence of SEQ ID NO:3." The Examiner's statement that "[o]n page 18234, col. 1, para. 3, Yabe et al. made antibodies against calumenin" does not indicate which of the four commercially available antibodies described in Yabe et al. allegedly anticipates the claimed antibodies. Furthermore, the Examiner has misinterpreted the statements made on page 18234, col. 1, para. 3 of Yabe et al. with regard to the antibodies described in the article. The Examiner purports that this passage is evidence that "Yabe et al. made antibodies against calumenin, anti-protein-disulfide isomerase antibody." What page 18234, col. 1, para. 3 of Yabe et al. actually

says is:

> *Antibodies*--Mouse anti-FLAG M2 antibody (Kodak), ***rabbit anti-protein-disulfide isomerase antibody (Stress Gen Biotechnologies)***, fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse IgG (H + L) (absorbed with human and rabbit serum) (Kirkegaard & Perry Laboratories Inc.), and rhodamine-conjugated affinity-purified goat anti-rabbit IgG (minimal cross-reaction to mouse IgG) (Biosource International) were used. [Emphasis added.]

On page 18235, column 2, paragraph 3 of Yabe et al., the authors describe how a ***commercially available antibody to protein-disulfide isomerase*** was used in conjunction with an anti-FLAG antibody to detect subcellular localization of a FLAG-C39 fusion protein (aka FLAG-calumenin).

> To determine the intracellular localization of the C39 protein, COS-7 cells transfected with FLAG-C39 were doubly stained with an anti-FLAG antibody and an anti-protein-disulfide isomerase antibody and observed under a confocal laser microscope. The staining profile of anti-FLAG antibody (Fig. 4B) was almost identical to that of anti-protein-disulfide isomerase antibody (Fig. 4A), showing diffuse ER staining patterns as reported previously for protein-disulfide isomerase, a well characterized protein in the ER (42). The identical staining patterns with both antibodies (Fig. 4C) indicate that the C39 protein is an ER-resident protein. Since the C39 protein has Ca^{2+} -binding ability and is localized in the ER lumen, we named the C39 protein calumenin. (Yabe et al. at page 18235, column 2, paragraph 3.)

Appellants submit that the antibodies used in these experiments were not directed against the 315 amino acid protein, calumenin, having 98.2% identity with SEQ ID NO:3. In fact, these commercially-available antibodies were directed against Bovine PDI and were purchased from Stress Gen Biotechnologies (see antibody description from Stress Gen Biotechnologies website and "Anti-PDI" product insert; Attachment 7). The immunogen used to make these antibodies is listed on the product insert as Bovine PDI from the sequence reported in M17596 in the GenBank database. M17596 (g163496) is a 510 amino acid protein having 22.8% identity (72/315 aa) to SEQ ID NO:3 (see GenBank records and CLUSTALW alignment; Attachment 8). Clearly, these antibodies do not anticipate the claimed subject matter. Furthermore, ***none*** of the four antibodies described in Yabe et al. anticipate the claimed subject matter.

Moreover, the Examiner fails to appreciate that the language of claim 10 requires that the antibodies ***specifically bind*** to SEQ ID NO:3. It is well understood in the art that an antibody which ***specifically binds*** to a polypeptide sequence binds to that polypeptide sequence ***only*** and does not bind to any other polypeptide sequences. Therefore, the antibodies taught in Yabe et al. which bind to calumenin do not ***specifically bind*** to SEQ ID NO:3 because they bind to other sequences as well. Accordingly, the antibodies recited in claim 10 are not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

II. Yabe et al. does not teach every aspect of the claimed invention either explicitly or impliedly

The Examiner has admitted that Yabe et al. teaches polypeptide sequences which are not 100% identical to the sequences to which the claimed antibodies bind. That is, the sequences taught by Yabe et al. are 98.2% identical to SEQ ID NO:3. (Final Office Action at page 3.) Furthermore, as is set forth above, no antibodies for the calumenin protein described in Yabe et al. are disclosed in the reference.

The Examiner's continued insistence that "with such high identity between calumenin and SEQ ID NO:3, it appears that the epitopic structure of one will be the same as the other and generate the same antibody" and that "the antibody taught in Yabe et al. is the same antibody claimed" (Final Office Action at page 4) is an attempt to determine the scope of the claim while ignoring the language of the claim. This rejection is further based on the allegation that the antibodies taught by Yabe et al. are within the scope of the claimed antibodies. Appellants strongly disagree with the Examiner's position.

Claim 10 recites "An isolated antibody which ***specifically binds*** to a polypeptide comprising the amino acid sequence of SEQ ID NO:3." (Emphasis added.) The claimed antibodies ***specifically bind*** to SEQ ID NO:3 such that those sequences are distinguished from all other sequences. As discussed above in § I., an antibody which ***"specifically binds"*** to SEQ ID NO:3 binds only to a polypeptide comprising SEQ ID NO:3 and will not bind to any other sequence. Appellants note that the claims do not recite an antibody which binds specifically to an epitope of a polypeptide, but rather an antibody which binds **specifically** to a **polypeptide** comprising the amino acid sequence of SEQ ID NO:3. The interaction of the antibody and the recited polypeptide is dependent on the epitope bound

by the antibody, but that does not mean that an antibody that binds specifically to an epitope on the recited polypeptide is the same thing as an antibody that binds specifically to the recited polypeptide. The antibodies recited by the claims bind **specifically** to the recited polypeptides. Since Yabe et al. teach polypeptides other than those recited by the claims; and the antibodies taught by Yabe et al. are raised against the immunogen Bovine PDI, the antibodies taught by Yabe et al. do not bind **specifically** to the recited polypeptides. The antibodies taught by Yabe et al. are excluded from the claimed antibodies because they do not bind **specifically** to the polypeptides recited in the claims.

Furthermore, antibodies which bind specifically to the polypeptide sequences recited in claim 10 can distinguish the differences between the recited polypeptide sequences and those disclosed in Yabe et al., even if the difference is only one amino acid. Evidence in support of this premise may be found in Abaza et al., J. Protein Chem. (1992) 11:433-444 (of record; copy supplied for the convenience of the Board as Attachment 4). As taught by Abaza et al., a single amino acid substitution outside the antigenic site on a protein effects antibody binding. This provides scientific support of Appellants' assertion that so long as there are differences, even just one amino acid residue, between the polypeptide sequences recited in claim 10 and those of the prior art, an antibody which specifically binds to the polypeptide sequences recited in claim 10 cannot be anticipated by the prior art. The Examiner's interpretation of Abaza et al. that "in the absence of sufficient guidance to a particular epitope and the structural context in which the epitope is found; [*sic*] it is highly unpredictable which other isolated polypeptides comprising a variant sequence of SEQ ID NO:2 would maintain the relevant antibody epitope(s)" (Final Office Action at page 5) is confusing. Since no variant sequences of SEQ ID NO:2 are described or claimed in the instant application, these remarks are irrelevant to the claimed subject matter. Appellants reiterate that Abaza et al. provides support for the ***specific binding*** of an antibody to a polypeptide and its ability to distinguish one polypeptide from another on the basis of even one amino acid difference between the polypeptide sequences. Therefore, because there are differences between the polypeptide sequences recited in claim 10 and those of the prior art, an antibody which specifically binds to the polypeptide sequences recited in claim 10 cannot be anticipated by the prior art. Accordingly, claims 10, 30, 31, 33, 36, 37 and 39-42 are not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

III. Bost et al., Bendayan, Li et al. and the Berg patent do not support the Examiner's position for rejection of claims 10, 30, 31, 33, 36, 37 and 39-42 for anticipation under 35 U.S.C. § 102(a).

In a further attempt to support the rejection of claims 10, 30, 31, 33, 36, 37 and 39-42 for anticipation under 35 U.S.C. § 102(a), the Final Office Action cites Bost et al. (Immunol. Invest. 1988; 17:577-586) and Bendayan (J. Histochem. Cytochem. 1995; 43:881-886) as evidence that antibodies that are highly specific can bind to "different molecules not related to the original antigen" (Final Office Action at page 5). Furthermore, the Examiner has cited Li et al. (Proc. Natl. Acad. Sci. USA 77:3211-3214, 1980) in support of the position that one must consider "dissociation of immunoreactive from other biological activities when constructing analogs" (Final Office Action at page 5). The Examiner further relies upon U.S. Pat. No. 6,210,670 (Berg) in support of this rejection; but does not make a specific argument for the use of this reference to support the rejection (Final Office Action at page 5). Appellants submit that none of these articles and/or patents have any relevance to the claimed subject matter.

Bost et al. demonstrate that polyclonal antibodies to the HIV envelope peptide LERILL could also recognize the LEHLLL epitope of IL-2. The Bendayan article describes crossreactivity in immunocytochemical applications of monoclonal antibodies directed to the Arg-Arg region of the human proinsulin molecule. When these two articles are examined in their proper context, neither article supports the Examiner's position that the claimed antibodies which specifically bind to SEQ ID NO:3 would bind to "different molecules not related to the original antigen." Both of these articles are based on studies of antibodies that are directed to very short stretches of amino acid sequences that could be found in many other full length proteins. The Bendayan article, in particular, is based on studies in tissues that were fixed and embedded in paraffin prior to sectioning and immunostaining for electron microscopic immunolabeling. This process changes the morphological state of the proteins present in the tissues which leads to a wider display of crossreactivity as described by the authors:

> The wider crossreactivity displayed by this antibody when used with morphological means could be based on the concept proposed (9) that the antibody recognizes not only a very particular amino acid sequence but also the three-dimensional conformation of this determinant. The processing of the tissues for morphological studies, which is known to affect the structure of proteins, may favor particular conformation(s) of similar

> determinants to resemble the domain present in the parent molecule. (Bendayan at page 886, paragraph 2.)

Appellants therefore submit that neither the Bost et al. nor the Bendayan article supports the Examiner's position that if "an antibody "cross-reacts", [*sic*] i.e., binds to more than one protein sequence, [it] does not mean that the antibody does not "specifically react" with both proteins" (Final Office Action at page 4). Rather, these papers indicate that if antibodies are directed to a small peptide and/or the morphology of the protein to be detected is altered prior to immunoassay, there is a greater likelihood that the antibodies will crossreact with proteins other than the parent molecule if those proteins share either highly homologous or identical short stretches of amino acid residues. This is not germane to what is claimed in the instant application.

Likewise, the Berg patent (U.S. Pat. No. 6,210,670) does not provide support for the Examiner's position regarding the use of the term ***"specifically binds."*** The Final Office Action purports that "Applicant's argument attempts to limit the term "specifically reacts" in a manner inconsistent with the well-known and art-recognized specificity of antibody interaction with epitopes defined by particular amino acid sequences." (Final Office Action at page 5.) The Berg patent teaches the generation of antibodies which bind to short stretches of an amino acid sequence that is common to 3 types of selectins (See the Berg patent at column 3, paragraph 3). These antibodies, as stated in the Summary of the Invention of the Berg patent, were designed to crossreact and bind to all 3 selectins because they only recognize those sequences which are common to all 3 selectins. Here, the Applicants in the Berg patent act as their own lexicographer in defining the term "specifically bind" (See the Berg patent at column 6, paragraph 7). Appellants in the instant application use the term ***"specifically binds"*** in the context of its ordinary meaning throughout the application; and there is no indication to the contrary. Therefore, the meaning of the term "specific binding," as used in the Berg patent, is not applicable to the same term as it is used in the instant application.

The courts have provided that dictionaries, encyclopedias and treatises may be used for interpretation of claim language:

> It has been long recognized in our precedent and in the precedent of our predecessor court, the Court of Customs and Patent Appeals, that dictionaries, encyclopedias and treatises are particularly useful resources to assist the court in determining the ordinary

and customary meanings of claim terms... (internal citations omitted) *Texas Digital Systems Inc. v. Telegenix Inc.*, 64 USPQ2D 1812 (Fed. Cir. 2002).

One must look to the ordinary dictionary definition of the term "specific." In order to put the language of the claims into the proper context, the term "specific" is defined in The American Heritage Dictionary, Second College Edition, as "...2. Pertaining to, ***characterizing or distinguishing a species***. 3. Special, ***distinctive, or unique***, as a quality or attribute. 4. Intended for, applying to, or acting upon a particular thing." (Emphasis added.) When the claims are read in light of this ordinary dictionary definition, it is clear that an "antibody which ***specifically binds*** to a polypeptide" is one which can ***uniquely distinguish*** that polypeptide from other polypeptide sequences. The Berg patent teaches antibodies which bind to short stretches of an amino acid sequence that is common to 3 types of selectins (See the Berg patent at column 3, paragraph 3) and also defines the term "specifically bind" for their own purposes in that particular patent application (See the Berg patent at column 6, paragraph 7). As used in the Berg patent, the definition of the term "specifically bind" cannot be construed as a "well-known and art-recognized specificity of antibody interaction with epitopes defined by particular amino acid sequences." Accordingly, the Berg patent does not provide support for the Examiner's allegation that Appellants' use of the term "specifically binds" is inconsistent with the art-recognized definition of the term (Final Office Action at page 5).

Appellants further submit that the teachings of Li et al. which support separate consideration of immunoreactive and biological activities when constructing analogs are not germane to the claimed subject matter and do not support the rejection on the basis as stated in the Final Office Action.

Once claim 10 (and therefore claims 30, 36 and 39) has been correctly characterized and considered in its proper context, the ancillary issues regarding antibodies in composition (claims 31, 37 and 40), having a label (claim 33), or being produced by either a Fab expression library or an immunoglobulin expression library (claims 41 and 42) become moot. For at least the above reasons, Appellants respectfully request that this rejection be overturned.

Issue 2--Claim rejections under 35 U.S.C. § 102(b)

Claims 10, 30, 31, 33, 36, 37 and 39-42 have been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Ozawa et al. (1993; J. Biol. Chem. 268:699-705; of record; copy supplied for the convenience of the Board as Attachment 9). The Final Office Action asserts that:

> Ozawa et al. teach reticulocalbin having 89.1% identity to SEQ ID NO:3. (Final Office Action at page 5.)
>
> Ozawa et al. made antibodies against reticulocalbin. Given that antibodies bind epitopic structures rather than sequences per se, and the identity between RCNδ and reticulocalbin is high, the antibody made by Ozawa et al. will also bind polypeptides having SEQ ID NO:3... (Final Office Action at page 4.)
>
> the antibodies were in composition (Claim 31, 37, 40)...the antibodies were labeled via conjugation with fluorescein isothiocyanate (Claim 33). Claims 41 and 42 are being considered to be anticipated as well because there appears to be no difference in the antibody made by a Fab expression library or and [sic] immunoglobulin expression library. (Final Office Action at page 4.)

Appellants strongly disagree with the Examiner's position for the following reasons.

First note that although the Response mailed on June 11, 2003 and received in the USPTO on June 13, 2003 contained evidence that the sequence the Examiner used to produce the alignment that was sent with the Office Action mailed March 11, 2003 is not the reticulocalbin sequence that was published on page 701 of the Ozawa et al. article, the Examiner did not acknowledge this in the Final Office Action mailed August 6, 2003. A copy of this evidence, which was submitted to the USPTO on June 11, 2003, is supplied for the convenience of the Board as Attachment 10. A careful visual comparison of the sequences reveals that the sequence used to produce the alignment labeled "RESULT 2" on page 2 of the document generated on Tue Feb 25 16:40:19 2003 (copy supplied for the convenience of the Board as Attachment 11) is 315 AA in length and begins with "MDLRQFLMC..." The reticulocalbin sequence published at page 701 of the Ozawa et al. article is 325 AA in length and begins with "MARGGRLGLALG..." Clearly, these are not the same sequences. Of particular note is that the 315 AA sequence used by the Examiner in the alignment labeled "RESULT 2" (Attachment 11) was submitted in ***October 1997*** and created in the TrEMBLrel. Database on January 1, 1998. The priority date of the instant application is ***August 8, 1997***.

Therefore, this sequence is not prior art to SEQ ID NO:3.

Appellants resubmit the attached CLUSTALW alignment (Attachment 10) of SEQ ID NO:3 (1601793CD1) with the reticulocalbin sequence published in Ozawa et al. (g220582). The attached alignment indicates that there is a 60% sequence identity (192/315 amino acids) between the two sequences--and the sequence identity is only that high because the CLUSTALW program inserted five gaps in SEQ ID NO:3 to effect the alignment. Additionally, Appellants submit that the rejection cannot withstand further scrutiny of the law under 35 U.S.C. § 102.

The law is clear with regard to the requirements for making a rejection under any subsection of 35 U.S.C. § 102:

> For a prior art reference to anticipate in terms of 35 U.S.C. § 102, every element of the claimed invention must be identically shown in a single reference." *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990).

Furthermore, the MPEP provides guidance for interpretation of the case law that has been generated with regard to 35 U.S.C. § 102:

> for anticipation under 35 U.S.C. 102, ***the reference must teach every aspect of the claimed invention either explicitly or impliedly***. Any feature not directly taught must be inherently present. (*MPEP 706.02* at page 700-21, Rev. 1, Feb. 2003) [Emphasis added.]

The polypeptide sequence taught by Ozawa et al. does not ***teach every aspect of the claimed invention either explicitly or impliedly***. The Examiner has alleged that the polypeptide sequence taught by Ozawa et al. (reticulocalbin) is 89.1% identical to SEQ ID NO:3 (RCNδ). (Final Office Action at page 4.) As noted above, the reticulocalbin taught by Ozawa et al. is only 60% identical to SEQ ID NO:3. It is, therefore, possible to make an antibody to SEQ ID NO:3 that does not bind the reticulocalbin taught in Ozawa et al. Such an antibody is recited in claim 10 as "An isolated antibody ***which specifically binds*** to a polypeptide comprising the amino acid sequence of SEQ ID NO:3." By **"specifically binding"** to SEQ ID NO:3, the claimed antibody must bind ***only*** to a polypeptide consisting of SEQ ID NO:3. Accordingly, so long as there are differences, even just one amino acid residue, between the amino acid sequence recited in claim 10 and that disclosed in Ozawa et al., an antibody can be produced that can specifically bind to the polypeptide recited in claim 10 and not that

of the reticulocalbin taught in Ozawa et al.

Evidence in support of this premise may be found in Abaza et al., J. Protein Chem. (1992) 11:433-444 (of record; Attachment 4). As taught by Abaza et al., a single amino acid substitution outside the antigenic site on a protein effects antibody binding. This provides scientific support of Appellants' assertion that so long as there are differences, even just one amino acid residue, between the amino acid sequence of claim 10 and those of the prior art, an antibody can be produced that can specifically bind to the polypeptide recited in claim 10 and not those of the prior art. Accordingly, given the extensive amino acid differences between SEQ ID NO:3 and reticulocalbin (at least 40%), one of skill in the art could produce an antibody which binds to the polypeptide recited in claim 10 alone and without cross-reactivity to other polypeptides even those which have extensive sequence identity to SEQ ID NO:3, which reticulocalbin does not have.

Ozawa et al. explicitly states that "[m]onospecific antibodies against reticulocalbin were prepared..." by immunizing rabbits with gel purified reticulocalbin fusion protein prepared in the maltose-binding protein (MBP) fusion vector, pMAL-c (Ozawa et al., page 700, column 1 paragraph 2 and column 2, paragraph 3). The nucleotide sequence used in the expression vector and the amino acid sequence of the fusion protein thus expressed are shown on page 701 at Figure 2 of Ozawa et al. The CLUSTALW alignment of SEQ ID NO:3 with the amino acid sequence of the fusion protein shown on page 701 at Figure 2 (Attachment 10) clearly shows that SEQ ID NO:3 and the amino acid sequence reported in Ozawa et al. are clearly not the same protein.

The Examiner's continued insistence that "[g]iven that antibodies bind epitopic structures rather than sequences per se, and the identity between RCN[δ] and reticulocalbin is high, the antibody made by Ozawa et al. will also bind polypeptides having SEQ ID NO:3, 90% identity to SEQ ID NO:3, biologically active fragments of SEQ ID NO:3, and immunogenic fragments of SEQ ID NO:3..." and that "the antibody taught in Ozawa et al. is the same antibody claimed" (Final Office Action at page 6) is an attempt to determine the scope of the claim while ignoring the language of the claim. This rejection is further based on the allegation that the antibodies taught by Ozawa et al. are within the scope of the claimed antibodies. Appellants strongly disagree with the Examiner's position.

First note that independent claim 10 does not recite "...90% identity to SEQ ID NO:3, biologically active fragments of SEQ ID NO:3, and immunogenic fragments of SEQ ID NO:3..." as

purported by the Final Office Action at page 6. Claim 10 recites "An isolated antibody **which specifically binds to a polypeptide** comprising the amino acid sequence of SEQ ID NO:3." (Emphasis added.) Appellants note that the claims do not recite an antibody which binds specifically to an epitope of a polypeptide, but rather an antibody which binds **specifically** to a **polypeptide** comprising the amino acid sequence of SEQ ID NO:3. The interaction of the antibody and the recited polypeptide is dependent on the epitope bound by the antibody, but that does not mean that an antibody that binds specifically to an epitope on the recited polypeptide is the same thing as an antibody that binds specifically to the recited polypeptide.

The courts have provided that dictionaries, encyclopedias and treatises may be used for interpretation of claim language:

> It has been long recognized in our precedent and in the precedent of our predecessor court, the Court of Customs and Patent Appeals, that dictionaries, encyclopedias and treatises are particularly useful resources to assist the court in determining the ordinary and customary meanings of claim terms... (internal citations omitted) *Texas Digital Systems Inc. v. Telegenix Inc.*, 64 USPQ2D 1812 (Fed. Cir. 2002).

One must look to the ordinary dictionary definition of the term "specific." In order to put the language of the claims into the proper context, the term "specific" is defined in The American Heritage Dictionary, Second College Edition, as "...2. Pertaining to, ***characterizing or distinguishing a species***. 3. Special, ***distinctive, or unique***, as a quality or attribute. 4. Intended for, applying to, or acting upon a particular thing." (Emphasis added.) When the claims are read in light of this ordinary dictionary definition, it is clear that an "antibody which ***specifically binds*** to a polypeptide" is one which can ***uniquely distinguish*** that polypeptide from other polypeptide sequences.

The antibodies recited by the claims bind **specifically** to the recited polypeptides. Since Ozawa et al. teach polypeptides other than those recited by the claims; and the antibodies taught by Ozawa et al. are raised against a gel purified reticulocalbin fusion protein prepared in the maltose-binding protein (MBP) fusion vector, pMAL-c (as shown in the amino acid sequence reported in Figure 2 of Ozawa et al.), the antibodies taught by Ozawa et al. do not bind **specifically** to the recited polypeptides. The antibodies taught by Ozawa et al. are excluded from the claimed antibodies because they do not bind **specifically** to the polypeptides recited in the claims.

Once claim 10 (and therefore claims 30, 36 and 39) has been correctly characterized and considered in its proper context, the ancillary issues regarding antibodies in composition (claims 31, 37 and 40), having a label (claim 33), or being produced by either a Fab expression library or an immunoglobulin expression library (claims 41 and 42) become moot. For at least the above reasons, Appellants respectfully request that this rejection be reversed.

Issue 3--Claim rejections under 35 U.S.C. § 112, second paragraph

Claims 36 and 39 have been rejected under 35 U.S.C. § 112, second paragraph for alleged indefiniteness as depending from claims directed to non-elected inventions. Appellants submit that, once product claim 10 is allowed, the method claims 35 and 38 from which claims 36 and 39 depend will be rejoined. Since the patentability of method claims 35 and 38 relies on the novelty and patentability of claim 10, and these claims do not expand the scope of the products recited in claim 10, claims 35 and 38 will be allowable upon rejoinder. Once this has been accomplished, claims 36 and 39 will depend from allowable method claims 35 and 38. Accordingly since Appellants are confident that the Board will overturn the 35 U.S.C. § 112, first paragraph and 35 U.S.C. § 102(a) and § 102(b) rejections against claim 10 and allow claim 10 after considering the arguments submitted above, Appellants respectfully submit that this rejection of claims 36 and 39 will be moot.

(9) CONCLUSION

Due to the urgency of this matter and its economic and public health implications, an expedited review of this appeal is earnestly solicited.

If the USPTO determines that any additional fees are due, the Commissioner is hereby authorized to charge Deposit Account No. **09-0108.**

This brief is enclosed in triplicate.

Respectfully submitted,

INCYTE CORPORATION

Date: 2/5/04

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Date: 05 February 2004

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Attachments:

1. Yabe et al.; July 18, 1997; J. Biol. Chem. 272:18323-18239
2. Bost et al.; Immunol. Invest. (1988); 17:577-586
3. Bendayan ; J. Histochem. Cytochem. (1995); 43:881-886
4. Abaza et al., J. Protein Chem. (1992) 11:433-444
5. Li et al.; Proc. Natl. Acad. Sci. (1980) USA 77:3211-3214
6. U.S. Pat. No. 6,210,670 (Berg)
7. Stress Gen Biotechnologies website and "Anti-PDI" product insert
8. g163496 GenBank records and CLUSTALW alignment with SEQ ID NO:3
9. Ozawa et al.; J. Biol. Chem. (1993); 268:699-705
10. CLUSTALW alignment of g220581 and SEQ ID NO:3, including NCBI documents for g220581 (3 pages)
11. alignment labeled "RESULT 2" on page 2 of the document generated on Tue Feb 25 16:40:19 2003

APPENDIX - CLAIMS ON APPEAL

10. An isolated antibody which specifically binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:3.

30. The antibody of claim 10, wherein the antibody is:

a) a chimeric antibody,

b) a single chain antibody,

c) a Fab fragment,

d) a $F(ab')_2$ fragment, or

e) a humanized antibody.

31. A composition comprising an antibody of claim 10 and an acceptable excipient.

33. A composition of claim 31, wherein the antibody is labeled.

36. An antibody produced by a method of claim 35.

37. A composition comprising the antibody of claim 36 and a suitable carrier.

39. A monoclonal antibody produced by a method of claim 38.

40. A composition comprising the antibody of claim 39 and a suitable carrier.

41. The antibody of claim 10, wherein the antibody is produced by screening a Fab expression library.

42. The antibody of claim 10, wherein the antibody is produced by screening a recombinant immunoglobulin library.

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Calumenin, a Ca^{2+}-binding Protein Retained in the Endoplasmic Reticulum with a Novel Carboxyl-terminal Sequence, HDEF*

(Received for publication, March 17, 1997)

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We have identified and characterized a cDNA encoding a novel Ca^{2+}-binding protein named calumenin from mouse heart by the signal sequence trap method. The deduced amino acid sequence (315 residues) of calumenin contains an amino-terminal signal sequence and six Ca^{2+}-binding (EF-hand) motifs and shows homology with reticulocalbin, Erc-55, and Cab45. These proteins seem to form a new subset of the EF-hand protein family expressed in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus. Purified calumenin had Ca^{2+}-binding ability. The carboxyl-terminal tetrapeptide His-Asp-Glu-Phe was shown to be responsible for retention of calumenin in ER by the retention assay, immunostaining with a confocal laser microscope, and the deglycosylation assay. This is the first report indicating that the Phe residue is included in the ER retention signal. Calumenin is expressed most strongly in heart of adult and 18.5-day embryos. The calumenin gene (*Calu*) was mapped at the proximal portion of mouse chromosome 7.

The endoplasmic reticulum (ER)[1] is involved in synthesis and modification of secretory and membranous proteins as well as resident proteins in the lumen of the ER, Golgi apparatus, or lysosomes (1). The ER is also known as the major Ca^{2+} storage compartment in eukaryotic cells. By pumping cytosolic Ca^{2+} into the ER lumen, cells keep their cytosolic concentration of free Ca^{2+} at extremely low levels so that they can use Ca^{2+} as intracellular signal (2). Besides, the ER itself needs luminal Ca^{2+} for its normal functions such as protein folding and protein sorting (3–6). Among many ER-resident proteins, endoplasmin (GRP94) (7, 8), Bip (GRP78) (9, 10), protein-disulfide isomerase (ERp59) (11, 12), and calreticulin (CRP55) (13) are Ca^{2+}-binding proteins that are associated with Ca^{2+}-dependent folding and maturation of secretory proteins in the ER lumen (14, 15). In addition, two Ca^{2+}-binding ER-resident proteins, reticulocalbin (16) and Erc-55 (17), have been isolated recently. They have multiple EF-hand motifs and constitute a new subset of the EF-hand superfamily, together with a homologous protein in the lumen of the Golgi apparatus, Cab45 (18). However, their physiological functions are still unknown.

ER-resident proteins generally carry a retention signal at their carboxyl terminus. This ER retention signal is first identified to be tetrapeptides, Lys-Asp-Glu-Leu (KDEL) in mammalian cells and His-Asp-Glu-Leu (HDEL) in yeast (19, 20). Soluble ER-resident proteins are trapped by binding to the KDEL receptor expressed in the cis-Golgi and retrieved to the ER (21–23). Further studies have demonstrated that HDEL and several variants of the KDEL sequence can also work as the ER retention signal in mammalian cells (24, 25). Comparison of variants of the KDEL sequence suggests that the replacement of Lys and Asp residues with other amino acid residues does not abolish the ER retention activity. However, the carboxyl-terminal two residues are considered to be critical because the third and fourth positions in all of the ER retention signals are Glu/Asp and Leu/Ile, respectively.

During the embryogenesis, the heart begins to beat already in the 8.5-day mouse embryo, whose heart is still a two-chambered tube with one atrium and one ventricle (26). Beating of cardiac myocytes is maintained by the strict regulation of their cytoplasmic Ca^{2+} concentration. To achieve this regulation, cardiac myocytes develop their specialized ER, called the sarcoplasmic reticulum, as the Ca^{2+} storage compartment and produce the rhythmical Ca^{2+} oscillation between the sarcoplasmic reticulum and the cytosol (27). Molecules involved in this Ca^{2+} oscillation are reported to be present very early in mouse cardiogenesis (28, 29).

Since we are interested in molecules involved in heart embryogenesis, we screened in a signal sequence trap library (30, 31) of mouse embryonic heart to isolate cDNAs encoding amino-terminal hydrophobic signal sequences. We here report cloning and characterization of cDNA encoding calumenin that binds Ca^{2+} and carries a new ER retention signal, HDEF, at the carboxyl terminus. Calumenin is a novel member of the reticulocalbin family, a new subset of the EF-hand superfamily in the ER. Calumenin is most strongly expressed in the heart of adult and 18.5-day embryos.

EXPERIMENTAL PROCEDURES

cDNA Cloning—Poly(A) RNA from approximately 100 hearts of 9.5-day postcoitus (dpc) mouse embryos was extracted with TRIzol reagent (Life Technologies, Inc.) and Oligotex™-dT30<Super> (Roche). cDNA was synthesized from 1.35 μg of poly(A) RNA using Super Script II (Life Technologies, Inc.). First strand cDNA was synthesized with 25 pmol of URPX3 primer: GAG-ACG-GTA-ATA-CGA-TCG-ACA-GTA-GCT-CGA-G*XXX-XXX-XXX-X* (where *X* represents one of the following: A, G, C, or T). After alkali lysis of RNA and the poly(A) tailing procedure, second strand cDNA was synthesized with 25 pmol of ESTN primer: CCG-CGA-ATT-CTG-ACT-AAC-TGA-$(T)_{17}$*XX*. Then cDNA of 400–800 base pairs were fractionated by agarose gel electrophoresis and subjected to polymerase chain reaction (PCR) using ExTaq (TaKaRa, Japan) and 25 pmol of ESP primer (CCG-CGA-ATT-CTG-ACT-AAC-TGA-TT) and Ad-P1 primer (GAC-GGT-AAT-ACG-ATC-GAC-AGT-AGG) under the following conditions for Thermal-Cycler (TaKaRa, Japan): 94 °C for 5 min and then 94 °C for 45 s, 52 °C for 60 s, and 72 °C for 2 min for 25 cycles and 72 °C for 10 min. After cloning cDNA unidirectionally between the *Eco*RI and *Xho*I sites of pSuc2t7F1ori vector, screening procedures were performed as described (31).

To clone full-length cDNA, the 3′-rapid amplification of cDNA ends

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U81829.

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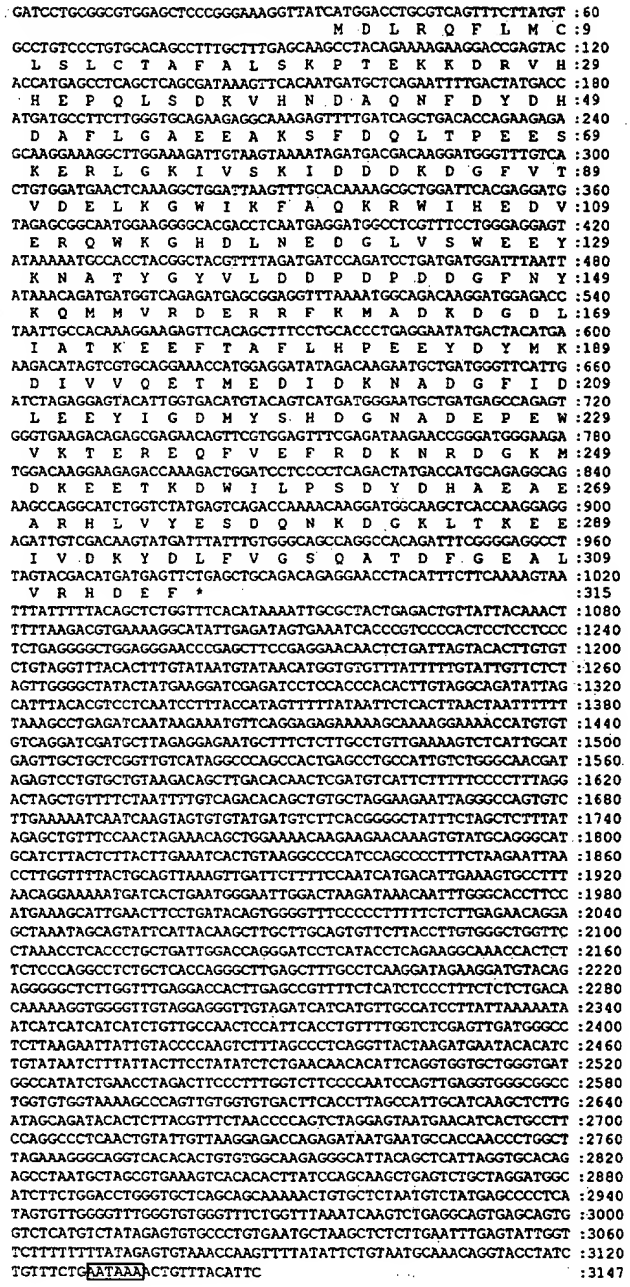
[1] The abbreviations used are: ER, endoplasmic reticulum; Bip, immunogloblin heavy chain binding protein; dpc, day(s) postcoitus; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

A



B

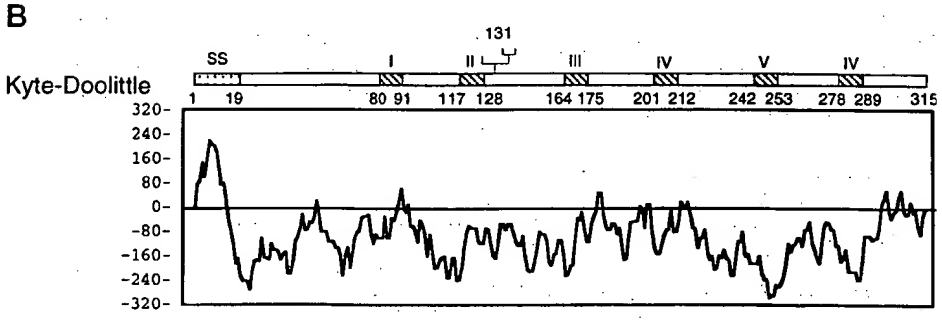


FIG. 1. **Nucleotide and predicted amino acid sequences of C39 cDNA.** *A*, positions of nucleotides (*upper row*) and amino acids (*lower row*) are shown at the *right*. cDNA sequence data is available from GenBank℠/EMBL/DDBJ under accession number U81829. The polyadenylation signal is *boxed*. *B*, schematic view and hydropathy plot of C39 protein. C39 protein has an amino-terminal signal sequence (*SS*), a predicted *N*-glycosylation site at position 131, and six EF-hand motifs. *Arabic numbers under* the *schema* indicate positions of amino acids at these landmarks. *Roman numbers* refer to EF-hand motifs. The hydropathy plot calculated by the algorithm of Kyte and Doolittle shows that the C39 protein has a typical amino-terminal signal sequence but no membrane anchor sequence.

(RACE) method was performed with LAtaq (TaKaRa). Mouse 13.5-dpc embryonic heart cDNA library in Uni-ZAP XR vector was used (Stratagene). C39N1b outer primer (biotinylated) (GTG-GAG-CTC-CCG-GGA-AAG-GTT-ATC-ATG) and C39N2 inner primer (CAT-GGA-CCT-GCG-TCA-GTT-TC) were designed as gene-specific primers and used with standard T7 primer (CGC-GTA-ATA-CGA-CTC-ACT-ATA-GGG-C). Conditions adopted for Perkin-Elmer 9600 were as follows: 95 °C for 2 min and then 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min for 25 cycles. The reaction mixture of first PCR with C39N1b and T7 primers was subjected to purification with M280 Dynabeads (Dynal) to decrease backgrounds due to nonspecific amplification by T7 primer. Second PCR was performed with C39N2 and T7 primers using the purified mixture.

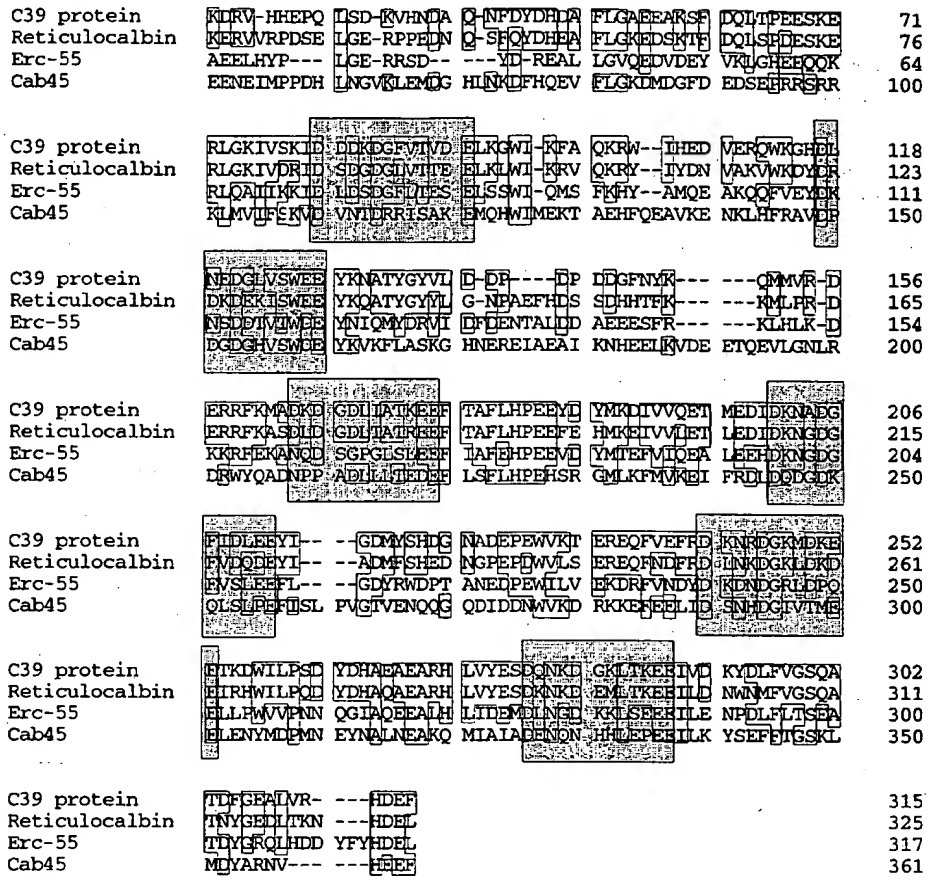


FIG. 2. **Alignment of C39, Erc-55, reticulocalbin, and Cab45.** All family members share significant homology even outside the EF-hand motif. EF-hand motifs are *shaded*. Amino acid residues identical to the C39 protein are *boxed*.

For sequencing, amplified cDNA was cloned into pGEM-T vector (Promega).

Sequencing was performed with an automated sequencer (model 373A; Applied Biosystems), and sequence analysis was done with the computer analysis program, GeneWorks (IntelliGenetics, Inc.). Homology search was performed with BLAST and FASTA, using GenBank® and EMBL as DNA data bases and PRF, PIR, and SwissProt as protein data bases. Motif search and localization analysis were performed on line at Prosite.

Expression and Purification of Proteins—To express proteins in mammalian cells, cDNAs were cloned in *Xba*I site of pEF-BOS expression vector (32), and their sequences were confirmed before assays. cDNAs of FLAG-calumenin (FLAG-C39), FLAG-calumenin-ΔHDEF, and FLAG-calumenin-rHDEL were constructed with PCR. FLAG epitope (8 amino acids; DYKDDDDK) was incorporated 5 amino acids downstream of the putative signal sequence cleavage site. The following primers were used for PCR: FLAG-calumenin primer, AAG-CCT-ACT--AGT-ATG-GAC-CTG-CGT-CAG-TTT-CTT-ATG-TGC-CTG-TCC-CTG--TGC-ACA-GCC-TTT-GCT-TTG-AGC-AAG-CCT-ACA-GAA-GAC-TAC--AAG-GAC-GAC-GAT-GAC-AAG-AAG-AAG-GAC-CGA-GTA-CAC-CA-T-GAG-C; calumenin-HDEF primer, AAG-CCT-ACT-AGT-TCA-GAA--CTC-ATC-ATG-TCG-TAC-TAA-GG; calumenin-ΔHDEF primer, AAG--CCT-ACT-AGT-TCA-TCG-TAC-TAA-GGC-CTC-CCC; and calumenin-rHDEL primer, AAG-CCT-ACT-AGT-TCA-CAA-CTC-ATC-ATG-TCG--TAC-TAA-GG. Proteins were purified from transfected cells according to the standard protocol for immunoprecipitation, using lysis buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris-HCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) and the anti-FLAG affinity gel (Eastman Kodak Co.).

Antibodies—Mouse anti-FLAG M2 antibody (Kodak), rabbit anti-protein-disulfide isomerase antibody (Stress Gen Biotechnologies), fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse IgG (H + L) (absorbed with human and rabbit serum) (Kirkegaard & Perry Laboratories Inc.), and rhodamine-conjugated affinity-purified goat anti-rabbit IgG (minimal cross-reaction to mouse IgG) (Biosource International) were used.

Other Methods—The procedures used to stain cells were essentially as described (18, 33). Stained cells were analyzed with a confocal scanning laser microscope (LSM410 UV, Carl Zeiss Inc.) following the protocol for double stain by fluorescein isothiocyanate and rhodamine. The Slow Fade Antifade kit (Molecular Probes, Inc.) was used to prevent photobleaching. Retention of calumenin in ER was assayed as follows. Cells transfected with FLAG-calumenin and FLAG-calumenin-ΔHDEF were further incubated for 4 h in serum-free medium. The media were then concentrated using Centricon-30 (Amicon). Cell extracts and concentrated media were analyzed by 12% polyacrylamide gel electrophoresis with SDS followed by Western blotting. The reverse transcriptase-PCR method. After first strand synthesis using random 9-mer, PCR was performed with C39N3 primer (GGA-AGA-TGG-ACA-AGG-AAG-AGA-CC) and C39C1b primer (biotinylated) (AGA-GTT-GTT-CCT-CGG-AAG-CTC-G). Transfection of COS-7 cells was performed using lipofectamine (Life Technologies) according to the manufacturer's protocol. Protein synthesis was stopped before protein purification and immunostaining assay by treating cells with 300 μM cycloheximide for 2 h (34). The filter for Northern blot was prepared as described (35) and hybridized using Quick-Hyb solution (Stratagene). The $^{45}Ca^{2+}$ binding assay was performed as described (36). Membranes of $^{45}Ca^{2+}$ binding assay and Northern blotting was analyzed using an image analyzer (BAS 2000, Fuji Film). Deglycosylation assay was done as described (18) and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. For Western blotting, ECL Western blotting detection reagents (Amersham Life Science, Inc.) was used. Chromosomal mapping of the calumenin gene was done as described (35, 37).

RESULTS

Cloning and Sequencing of a Novel cDNA C39—4.4×10^5 yeast transformants from 9.5 dpc embryonic heart cDNA library were screened, and 386 positive clones were obtained by the signal sequence trap method described previously (30, 31). Among these, nucleotide sequences of 17 clones were not reported in any mammalian species, 15 clones were homologous to sequences reported in mouse or other mammals and the rest were redundant clones. One of the novel clones (C39) was picked up for further studies because its mRNA was most strongly expressed in heart (see below). The full-length cDNA of C39 was isolated from a 13.5 dpc embryonic heart cDNA library by the 3′-RACE method. Nucleotide sequences of two independent clones were determined to avoid sequence errors

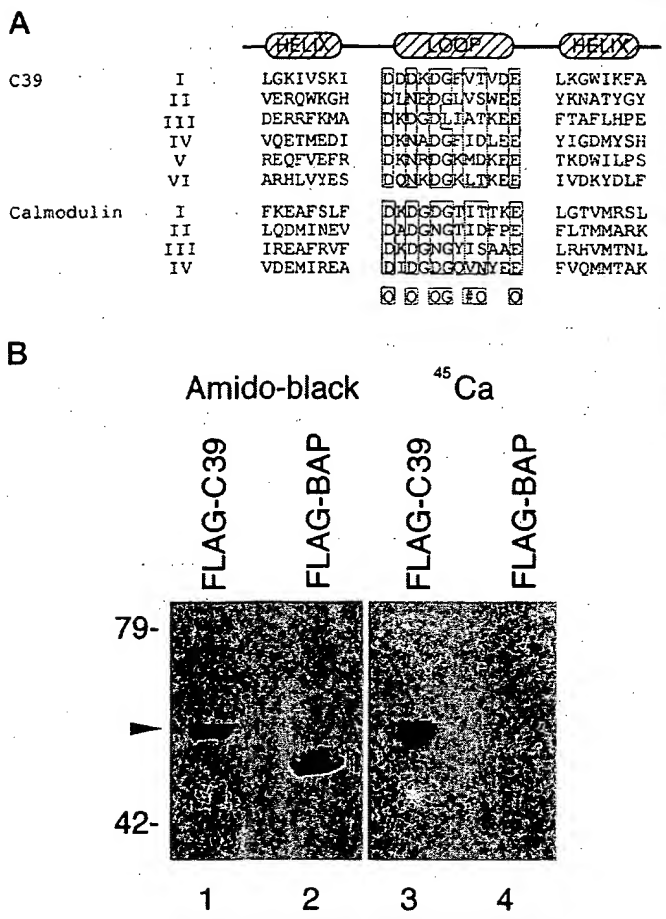


FIG. 3. **C39 encodes a Ca^{2+}-binding protein.** *A*, EF-hand motifs of the C39 protein are compared with those of calmodulin. The EF-hand motif is defined as the sequence consisting of the loop domain and two flanking helices. Consensus amino acid residues in the loop domain are *boxed*. # and O indicate a consensus hydrophobic amino acid and amino acid with the oxygen-containing side chain, respectively. Glycine (*G*) in the loop domain is often but not necessarily conserved in active EF-hand motifs. *B*, a $^{45}Ca^{2+}$ binding assay was performed as described (36). The purified FLAG-C39 fusion protein (*lanes 1* and *3*) and the FLAG-bacterial alkali phosphatase (*FLAG-BAP*) fusion protein (*lanes 2* and *4*) were electrophoresed in 12% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and incubated with $^{45}Ca^{2+}$. After washing, the filter was exposed in an image analyzer (^{45}Ca; *lanes 3* and *4*). The filter was also stained with Amido Black after the exposure (*Amido Black*; *lanes 1* and *2*). The *arrowhead* indicates the FLAG-C39 fusion protein.

and shown to encode a 315-amino acid protein (Fig. 1*A*). The translation start site was assigned at nucleotide positions 36–38 because of the presence of an NH_2-terminal signal sequence and the comparison with other family members to be described below.

The C39 Protein Is a Ca^{2+}-binding Protein—cDNA sequence showed that the C39 protein does not have any other hydrophobic stretch long enough to anchor the protein in the membrane (Fig. 1*B*). The C39 protein carries six potential Ca^{2+}-binding EF-hand motifs and a putative *N*-glycosylation site. Homology search with the BLAST and FASTA computer program revealed the presence of many Ca^{2+}-binding proteins homologous to the C39 protein in the EF-hand region. Among these, reticulocalbin (16) and Erc-55 (17) in the ER and Cab45 (18) in the Golgi apparatus had homology in size and sequence to the C39 protein even outside of the EF-hand region (Fig. 2). These proteins appear to form one subfamily of Ca^{2+}-binding proteins.

Each EF hand in the C39 protein has the general feature for high affinity Ca^{2+}-binding according to Kretsinger's rule (38);

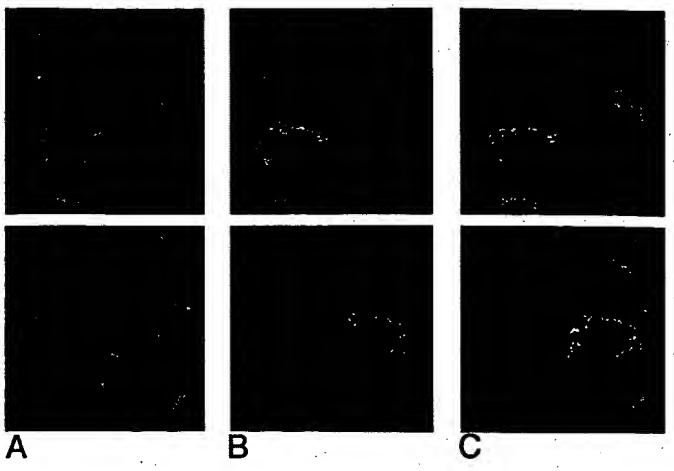


FIG. 4. **Localization of C39 protein by immunostaining.** COS-7 cells, which were transfected with pEF-BOS-FLAG-C39, were doubly stained with anti-FLAG antibody (*B*; visualized with a secondary fluorescein isothiocyanate-conjugated antibody) and anti-protein-disulfide isomerase antibody (*A*; visualized with a secondary rhodamine-conjugated antibody) after 2 h of treatment with 300 μM cycloheximide. Staining profiles were observed using a confocal scanning laser microscope. *C* is an overlap image of *A* and *B*.

the presence of the helix-loop-helix motif in which 5 oxygen-containing residues and the central glycine are conserved for coordination of Ca^{2+} binding (Fig. 3*A*). Although the central glycine of the third motif is replaced by leucine, the same replacement was seen in other Ca^{2+}-binding proteins (39, 40) and considered to retain the Ca^{2+}-binding activity by the secondary structure prediction of computer analysis (41).

To test whether six EF hands in the C39 protein really have Ca^{2+}-binding ability, the $^{45}Ca^{2+}$ binding assay was performed as described (36). Strong signal was detected in the FLAG-C39 fusion protein but none in the FLAG-bacterial alkali phosphatase fusion protein (Fig. 3*B*). The positive band was approximately 57 kDa in size, which was equal to the size of the FLAG-C39 fusion protein detected by Amido Black staining, indicating that EF hands in the C39 protein indeed have Ca^{2+} binding ability.

Subcellular Localization of the C39 Protein—To determine the intracellular localization of the C39 protein, COS-7 cells transfected with FLAG-C39 were doubly stained with an anti-FLAG antibody and an anti-protein-disulfide isomerase antibody and observed under a confocal laser microscope. The staining profile of anti-FLAG antibody (Fig. 4*B*) was almost identical to that of anti-protein-disulfide isomerase antibody (Fig. 4*A*), showing diffuse ER staining patterns as reported previously for protein-disulfide isomerase, a well characterized protein in the ER (42). The identical staining patterns with both antibodies (Fig. 4*C*) indicate that the C39 protein is an ER-resident protein. Since the C39 protein has Ca^{2+}-binding ability and is localized in the ER lumen, we named the C39 protein calumenin.

The HDEF Sequence Is a Novel ER Retention Signal—To examine whether the C-terminal tetrapeptide HDEF of calumenin can serve as a novel intracellular retention signal, we constructed two expression vectors: FLAG-calumenin, a fusion protein of FLAG epitope and calumenin, and FLAG-calumenin-ΔHDEF, a fusion protein of the FLAG epitope and calumenin lacking its C-terminal HDEF sequence. FLAG epitopes were incorporated 5 amino acids downstream of the putative signal sequence cleavage site in each construct. COS-7 cells were transfected by these constructs, and concentrated media and cell extracts were analyzed by Western blotting. Most of the FLAG-calumenin-ΔHDEF protein was secreted into the medium, while almost all of the FLAG-calumenin protein was

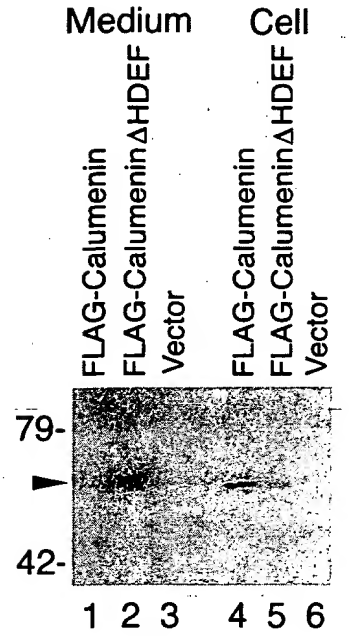


FIG. 5. **HDEF works as a intracellular retention signal.** A retention assay was performed using COS-7 cells transfected with pEF-BOS-FLAG-calumenin, pEF-BOS-FLAG-calumenin-ΔHDEF and pEF-BOS (control). After transfection, cells were washed and chased for a further 4 h in serum-free media. Then media were concentrated and analyzed by Western blotting together with cell extracts. FLAG-calumenin-ΔHDEF was secreted (*lane 2*), while almost all of the FLAG-calumenin was retained in cells (*lane 1*). Different amounts of proteins in cells may be due to the secretion of FLAG-calumenin-ΔHDEF (*lanes 4* and *5*). The *arrowhead* indicates the FLAG-calumenin fusion protein.

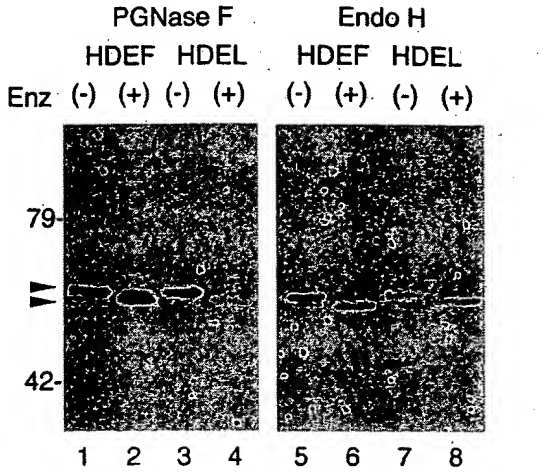


FIG. 6. **Calumenin is a glycosylated protein in the ER.** Transfected cells were treated with 300 μM cycloheximide for 2 h to stop further protein synthesis. The FLAG-calumenin (HDEF) and FLAG-calumenin-rHDEL (HDEL) proteins were purified and subjected to endoglycosidase H and *N*-glycosidase F digestion for 1 h at 37 °C. The *upper band* indicates glycosylated proteins, and the *lower band* indicates deglycosylated forms. *Enz*, enzyme.

retained in the cell (Fig. 5). These results indicate that HDEF is essential to maintain calumenin within the cell.

We further examined intracellular localization of calumenin by treatment with deglycosidases. Proteins translocated into the ER are exposed to core glycosylation. At this moment, glycosylated proteins are sensitive to the deglycosidase endoglycosidase H, which cleaves high mannose oligosaccharides on proteins. However, once proteins are transported into the medial Golgi and modified by Golgi-mannosidase II, they become resistant to endoglycosidase H. However, Golgi proteins are still sensitive to *N*-glycosidase F, a glycosidase whose activity is not disturbed by the following carbohydrate modifications in the medial- and trans-Golgi. Calumenin had one putative *N*-linked glycosylation site. Affinity-purified FLAG-calumenin protein was incubated with either endoglycosidase H or *N*-glycosidase F and subjected to Western blotting with the anti-FLAG M2 antibody. We also performed the assay against FLAG-calumenin-rHDEL, whose C-terminal tetrapeptide HDEF was replaced with HDEL, known as the ER retention signal. Both FLAG-calumenin and FLAG-calumenin-rHDEL showed strong bands at 57 kDa without the glycosidases (Fig. 6, *lanes 1*, *3*, *5*, and *7*). Digestion with either endoglycosidase H or *N*-glycosidase F shifted the size of the strong bands to 52 kDa (*lanes 2*, *4*, *6*, and *8*). We consider that 57-kDa bands correspond to the glycosylated form of calumenin, and 52-kDa bands correspond to the deglycosylated form. Calumenin is indeed translocated and glycosylated in the ER and not modified in the Golgi apparatus, because Endo H completely cleaved oligosaccharides on FLAG-calumenin (*lanes 2* and *6*) and FLAG-calumenin-rHDEL (*lanes 4* and *8*). These results indicate that calumenin resides in the ER and that the C-terminal tetrapeptide HDEF works as the ER retention signal like HDEL (16, 17).

Expression and Chromosomal Localization of the Calumenin Gene—Northern blotting analysis showed that calumenin was expressed ubiquitously in all tissues examined. However, the expression of calumenin was strong especially in heart and lung. Compared with expression in adult heart, expression in 18.5-dpc heart was slightly stronger (Fig. 7*A*). The reverse transcriptase-PCR method showed that calumenin mRNA was already expressed as early as 8.5 dpc (Fig. 7*B*).

To determine the chromosomal localization of the calumenin gene (*Calu*),[2] strain distribution patterns of restriction fragment length polymorphisms of the calumenin gene were determined in 24 independent recombinant inbred strains derived from crosses between AKR/J and DBA/2J (AXD) (Table I). Analysis of the distribution pattern revealed the linkage of the calumenin gene with markers located at the proximal region of chromosome 7 (Fig. 8).

DISCUSSION

A Novel Ca^{2+}-binding Protein in the ER Lumen—We isolated and characterized a novel Ca^{2+}-binding protein calumenin from mouse embryonic heart. Calumenin is located in the ER and homologous to previously reported Ca^{2+}-binding proteins such as reticulocalbin and Erc-55 in the ER (16, 17), and Cab45 (18) in the Golgi apparatus. Since all of these proteins including calumenin have six EF hands and Ca^{2+}-binding activity, they constitute one subset of the EF-hand superfamily. A data base search also revealed that cDNA (λSCF13) cloned as DNA supercoiling factor of silkworm (43) also belongs to this family. The deduced amino acid sequence of λSCF13 has an amino-terminal signal sequence, six EF hands, and the C-terminal HDEF sequence, which we proved to be the ER retention signal.

Although many ER Ca^{2+}-binding proteins have been reported so far, their functions are largely not yet well understood. Two possible functions of ER Ca^{2+}-binding proteins have been suggested. First, calreticulin, one of the Ca^{2+}-binding proteins in the ER, is reported to regulate the capacity of Ca^{2+} in the ER (44, 45) as well as to have chaperone function (46, 47). Another luminal Ca^{2+}-binding protein, calsequestrin, is also reported to regulate Ca^{2+} flow from the ER (48, 49). These reports suggest that Ca^{2+} fluxes may be regulated somehow by

[2] The gene name *Calu* for calumenin has been approved by Lois J. Maltais, the Nomenclature Coordinator for the Mouse Genome Data base (The Jackson Laboratory, Bar Harbor, ME).

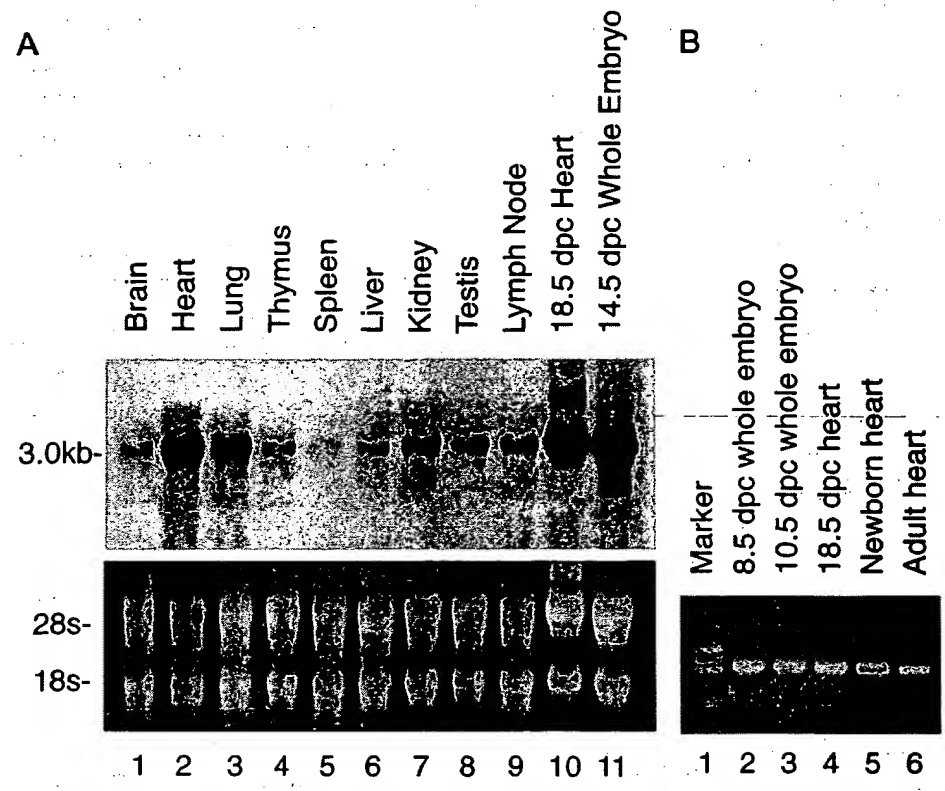


FIG. 7. **Expression pattern of calumenin.** *A*, Northern blotting of mouse RNA with calumenin cDNA as probe. 20 μg of total RNA from various mouse tissues were applied to each well (*lower column*), and Northern blotting was performed using a 512-base pair C39 fragment obtained in the initial screening as a probe (*upper column*). *B*, reverse transcriptase-PCR was performed using 1 μg of total RNA and calumenin-specific primers. After 28 cycles of PCR, products were electrophoresed and stained with ethidium bromide.

TABLE I

Strain distribution patterns of calumenin (Calu) and closely linked markers in RI strains AXD

Restriction fragment length polymorphisms were found by *Apa*I cleavage of the calumenin in AKR/J (3.8 kilobase pairs) and DBA/2J (4.8 kilobase pairs). Determination and analysis of the restriction fragment length polymorphism distribution pattern among recombinant inbred strains derived from AKR/J and DBA/2J were performed as described (35, 37). Strain-specific alleles are abbreviated as A (AKR/J) and D (DBA/2J). Unknown alleles that differ from A and D are identified with U. Putative crossing over points are indicated by X.

Locus	AXD recombinant inbred strain number																						
	1	2	3	6	7	8	9	10	11	12	13	14	15	16	18	20	21	22	23	24	25	26	27	28
Pmv4	A	D	A	A	D	D X	D	D X	D	D X	D	A	A	A	A	D	A	A	A	A	A	D	D	D
Calu	A	D	A	A	D	A	D	A	D	A	D	A	A	A	A	D	A	A	A	A	A	D	D	D
Emv11	A	D	A X	A	D	A	D	A	D	A	D X	A	A	A	A	D	A	A	A	A	A X	D	D	D
Ckmm	A	D	D	A	D	A	D	A X	D	A	A X	A	A	A	A	D X	A	A	A	A	D	D X	D	D
Pmv29	A	D	D X	A	D	A	D	D X	D	A	D X	A	A	A	A	A	A	A	A	A	D	A X	D	D
Mag	A	D	A	A	D	A	D	A	D	A	A	A	A	A	A	A	A	A	A	A	D	D X	D	D
Upk1a	A	D	A	A	D	A	D	A	D	A	A	A	A	A	A	A	A	A	A	A	U	A	D	D
Abpa	A	D	A	A	D	A X	D	A	D	A X	A	A	A	A	A	A	A	A	A X	A	D	A	D	D
Odc-rs6	A	D	A X	A	D	D	D	A	D	D	A	A	A	A	A	A	A	A	D	A	D	A	D	D
Xmv30	A	D	D	A	D	D	D	A	D	D	A	A	A	A	A	A	A	A	D	A	D	A	D	D
Tam1	A	D	D	A	D	D	D	A	D	D	A	A	A	A	A	A	A	A	D	A	U	A	D	D

Ca^{2+}-binding proteins located in the luminal side of the ER. Second, Ca^{2+} in the ER is reported to be necessary for normal functions of the ER such as protein folding and protein sorting (3–6), suggesting the existence of ER-resident molecules whose function is regulated by Ca^{2+}. In fact, Bip, a Ca^{2+}-binding protein in the ER, is reported to function in the Ca^{2+}-dependent manner (50).

Further study is needed to determine whether or not calumenin is involved in either of the two possible functions. Recently, interaction molecules of Erc-55 have been reported: the E6 protein of papilloma virus, which has p53-independent tumorigenic activity (51), and taipoxin, which blocks neuromuscular transmission at the presynaptic site (52). As discussed above, calumenin belongs to the same subset of the EF-hand superfamily as Erc-55. Thus, the search for proteins that interact with calumenin could provide a clue to elucidate biological functions of this subset.

A Novel ER Retention Signal—We report a novel carboxyl-

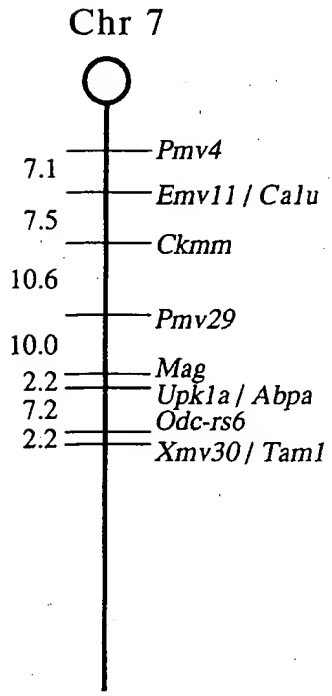


FIG. 8. **Chromosome map surrounding calumenin locus on mouse chromosome 7.** The position of the calumenin locus (designated by *Calu*) is shown on chromosome 7 based on data from AXD strains (Table I). The centromere is indicated by a *circle*. Recombination distances in centimorgans are shown at the *left* of the chromosome.

terminal tetrapeptide HDEF of calumenin as a new ER retention signal to prevent proteins from being secreted and to keep them in the ER. We observed that the HDEF sequence had the intracellular retention activity (Fig. 5). The deglycosylation assay (Fig. 6) and the immunostaining profile (Fig. 4) suggest that the HDEF sequence works as the ER retention signal. The C-terminal tetrapeptide KDEL is the first ER retention signal reported in mammalian cells (20). The C-terminal tetrapeptide HDEL, which was originally identified in yeast, was also found to work as the ER retention signal in mammalian cells (16, 17). Other reports show that the first and second positions of the C-terminal tetrapeptide are not strongly conserved to maintain the ER retention activity. In contrast, replacement of the third and fourth residues is strictly restricted. The third position requires an acidic residue such as Glu or Asp, and the fourth position requires a hydrophobic residue containing the aliphatic side chain such as Leu or Ile, which cannot be replaced by either Val or Ala (24, 25). Since Cab45, a soluble protein in the Golgi apparatus has HEEF sequence at the C terminus, this tetraresidue sequence was presumed to serve as the Golgi retention signal. The C-terminal Phe, a hydrophobic residue containing an aromatic side chain, was thought to work somehow differently from Leu (18). However, since our study clearly showed that the HDEF sequence worked as the ER retention signal, the retention signal to the Golgi apparatus may be localized in another portion of Cab45.

Proteins with ER retention signals such as KDEL are retrieved from Golgi. The convincing mechanism of this retrieval is that such proteins interact with the KDEL receptor distributing in the cis-Golgi and are retrieved to the ER (22, 23). We showed that the C-terminal HDEF sequence worked as the ER retention signal, but further investigation is needed to determine whether HDEF interacts with the KDEL receptor or with an unknown receptor.

Chromosomal Mapping of the Calumenin Gene (Calu)—The calumenin gene was mapped on the proximal portion of mouse chromosome 7. This region is well conserved between human and mouse, and the syntenic comparison suggests that this region corresponds to human 19q13.2–13.3 (53), at which the gene for progressive familial heart block type I, autosomal dominantly inherited cardiac bundle-branch disorder, is mapped (54). Since the causal gene of this disease has not been determined so far, further investigation is needed to determine whether the calumenin gene is involved in this disease.

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ANTIBODIES AGAINST A PEPTIDE SEQUENCE WITHIN THE HIV ENVELOPE PROTEIN CROSSREACTS WITH HUMAN INTERLEUKIN-2

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ABSTRACT

Recent investigations have identified a homologous sequence between the lymphokine interleukin 2 (IL-2) and the envelope protein of HIV. This homology is one of six amino acids corresponding to interleukin-2 (IL-2) residues 14-19 (Leu-Glu-His-Leu-Leu-Leu) and to the carboxy terminal six amino acids of HIV envelope protein gp41 (Leu-Glu-Arg-Ile-Leu-Leu). Thus, it is conceivable that an anti-HIV antibody response would generate antibodies which would crossreact with IL-2. We show here that the two peptides are recognized by the immune system as being almost identical. More importantly, antibodies against the HIV envelope peptide bind IL-2. Thus, these studies are the first step in investigating what could be characterized as an HIV-induced autoimmune response, that is, the induction of antibodies to the HIV envelope protein which also crossreact with IL-2.

INTRODUCTION

Recently, an amino acid sequence homology between the human immunodeficiency virus (HIV) envelope protein and human interleukin-2 (IL-2) was described (1,2). The homology is six amino acids in length and corresponds to the carboxy terminal six amino acids of the envelope protein, gp41 (3-5), and amino acids 14 to 19 of the secreted IL-2 molecule (6). For human IL-2, the sequence Leu-Glu-His-Leu-Leu-Leu (LEHLLL) shows a striking homology to a sequence in the HIV envelope protein Leu-Glu-Arg-Ile-Leu-Leu (LERILL). This six amino acid sequence is highly conserved among the viral strains isolated thus far (2-5;7-9), so there would seem to be some selective advantage for its presence.

One possible advantage might be the stimulation of an anti-LERILL antibody response upon viral infection which was crossreactive with IL-2. Since IL-2 is necessary for immune competence (10), antibodies against this protein might be deleterious. In fact, rabbit antisera against amino acids 7-21 of IL-2 (which includes LEHLLL) neutralized IL-2 biological activity (11). These studies along with others (1,12,13) demonstrate that the sequence LEHLLL is involved in binding of IL-2 to its receptor. Clearly, this sequence is important for the mediation of IL-2 biological activity.

The purpose of this study is to determine whether antibodies against the HIV envelope peptide, LERILL, recognize the IL-2 sequence, LEHLLL, and ultimately whether these antibodies will recognize the intact IL-2 molecule.

METHODS

Immunogen Preparation and Immunization

Each peptide was synthesized on a 9500 Solid Phase Peptide Synthesizer (Biosearch, San Rafael, CA) using t-boc chemistry (14). Peptides were cleaved from the resin using a hydrogen fluoride cleavage apparatus (Peninsula Laboratories, Belmont, CA) at 0°C in the presence of 10% anisole. After ethyl acetate precipitation, peptides were dissolved in 0.5 M acetic acid, lyophilized and then subjected to high pressure liquid chromatography (HPLC) purification using a C-18 150A Dynamax HPLC column (Rainin, Woburn, MA). Purity of peptides was assessed by the appearance of a single symmetrical peak on HPLC and amino acid compositional analysis. Peptides were greater than 98% pure using these criteria. Peptides were stored under nitrogen at 4°C until used.

For immunization, 5 mg of each peptide were coupled to 5 mg of keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA) using 10 mM glutaraldehyde in 0.05 M phosphate buffered saline (PBS, pH 7.2) for 4 hours at room temperature. After overnight dialysis against PBS, conjugates were aliquoted and stored at -20°C. Coupling efficiency was always between 65% and 80% as determined using the addition of ^{125}I-tyrosine containing analogs of each peptide to the coupling reaction to determine conjugation ratios.

Sprague Dawley rats (3 per group) were immunized subcutaneously and intraperitoneally with a total of 300 μg of peptide-KLH conjugate emulsified in incomplete Freund's adjuvant. Rats were boosted every 10 days for a total of four immunizations. Ten days after the final injection, rats were bled from the tail vein under ether anesthesia, and sera collected.

Enzyme-linked immunosorbant assay (ELISA)

To test antibody reactivity, peptides were adsorbed onto Nunc immuno-plates (Nunc, Thousand Oaks, CA) in 0.05 M carbonate buffer (pH 9.2) at 100 μg per well. After 18 hours at 4°C, nonadsorbed peptide was washed off with buffer (PBS-0.5% BSA-0.02% Tween-20) and the plates blocked for 2 hours with PBS-2% BSA. Sera or purified antibody was diluted in buffer, and varying dilutions were incubated on peptide coated plates for 90 minutes. After washing 3 times with buffer, a 1:1000 dilution of an anti-rat IgG antibody (Boehringer Mannheim, Indianapolis, IN) was added for 60 minutes. Unbound secondary antibody was removed by washing three times with buffer, and bound antibody was detected using nitrophenyl phosphate (Sigma, St. Louis, MO) in carbonate buffer (pH 9.2) as the substrate.

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Peptide Synthesizer
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Antibody reactivity was determined by reading the absorbance at 405 nm using a model 2550 EIA reader (Bio-Rad, Richmond, CA) approximately 30 minutes after substrate addition.

To assure specificity of binding, three different controls were used. First, sera from rats immunized with KLH only were used to demonstrate any nonspecific binding to the peptide. Second, binding of antisera to peptide coated wells was blocked with 1 mM soluble peptide. For blocking, peptides were conjugated to BSA via glutaraldehyde, and conjugation ratios were determined using ^{125}I-tyrosine containing derivatives of each peptide. Finally, irrelevant peptides (i.e., MAYKE and SATCTI) were coated onto plates to assess nonspecific binding of the antisera.

To test antibody reactivity against IL-2, the same ELISA procedure described above was used. Instead of peptides, recombinant IL-2 (AMGEN, Thousand Oaks, CA) was adsorbed onto Nunc high protein binding microwell modules (Nunc, Thousand Oaks, CA) at a coating concentration of 200 ng per well in carbonate buffer (pH 9.2). A heterologous rabbit anti-human IL-2 antibody (Genzyme, Boston, MA) was used for blocking experiments.

Affinity Purification of Antibodies

Antibodies to peptides were specifically purified using peptide conjugated affinity columns. Fifty mg of each peptide were dissolved in 0.05 M HEPES buffer (pH 8.0) and reacted with 10 ml of Affi-gel 10 (Bio-Rad, Richmond, CA) for 18 hours at 4°C. Unreacted groups were subsequently blocked with excess glycine. Sera from immunized rats were diluted 1:2 with PBS-0.05% aprotinin and passed over the appropriate affinity column. Bound antibodies were eluted with 0.1 M glycine-0.1 M NaCl (pH 3.0), subsequently neutralized and then dialyzed extensively against PBS. Antibody concentration was determined by absorbance at 280 nm (Shimadzu UV-160, Kyoto, Japan). Greater than 93% of protein binding to the affinity column was immunoglobulin as determined by densitometric scans of SDS-polyacrylamide gels.

RESULTS

Antibodies Against the HIV Envelope Peptide, LERILL, Also React with the IL-2 Sequence, LEHLLL

To demonstrate that antibodies against the HIV envelope peptide LERILL could also recognize the LEHLLL epitope of IL-2, groups of rats were immunized with either peptide. Figure 1 shows that when ELISA plates were coated with the peptide LEHLLL, antisera against LEHLLL specifically bound this peptide as would be expected. In fact, all three rats had titers to the peptide greater than 1:60,000. Controls demonstrated the specificity of this binding. Not only did anti-KLH sera not react, but greater than 90% of the antisera binding could be blocked with 1 mM soluble peptide conjugated to BSA. Irrelevant peptide-BSA conjugates blocked less than 8% of the binding. Furthermore, plates coated with irrelevant peptides reacted less than 5% as well as LEHLLL-coated plates.

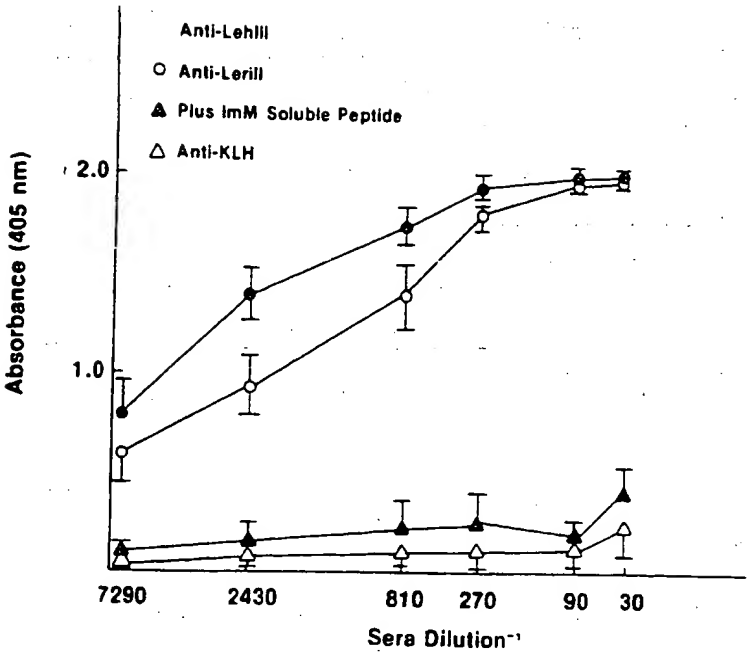


FIGURE 1

Reactivity of antisera against LEHLLL and LERILL on plates coated with LEHLLL. Sera from rats (3 per group) immunized with LEHLLL (●—●), LERILL (○—○) or KLH (△—△) were assayed for reactivity against LEHLLL using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM LEHLLL or 1 mM LERILL (▲—▲) to anti-LEHLLL sera during the reaction. Nonspecific binding is shown using anti-KLH sera. Each point represents the mean reactivity of three different rats ± standard deviations.

While it is clear that the ELISA reaction was specific, the important data in Figure 1 is the reactivity of sera from rats immunized with the HIV envelope peptide, LERILL, with the IL-2 peptide, LEHLLL. While the reactivity of antisera to LEHLLL is qualitatively higher, there is no statistically significant difference when antisera to LERILL is used. Furthermore, either soluble BSA-LEHLLL or BSA-LERILL could block greater than 90% of the binding.

Similar results were obtained when the reciprocal experiment was carried out. Plates coated with the HIV envelope peptide, LERILL, reacted specifically and with high titer (greater than 1:60,000) to sera from rats immunized with this peptide (see Figure 2). Once again, the reactivity using antisera against the homologue, LEHLLL, was not significantly different. Taken together, the results from Figures 1 and 2 conclusively demonstrate that the two peptides, LEHLLL and LERILL, are recognized by the

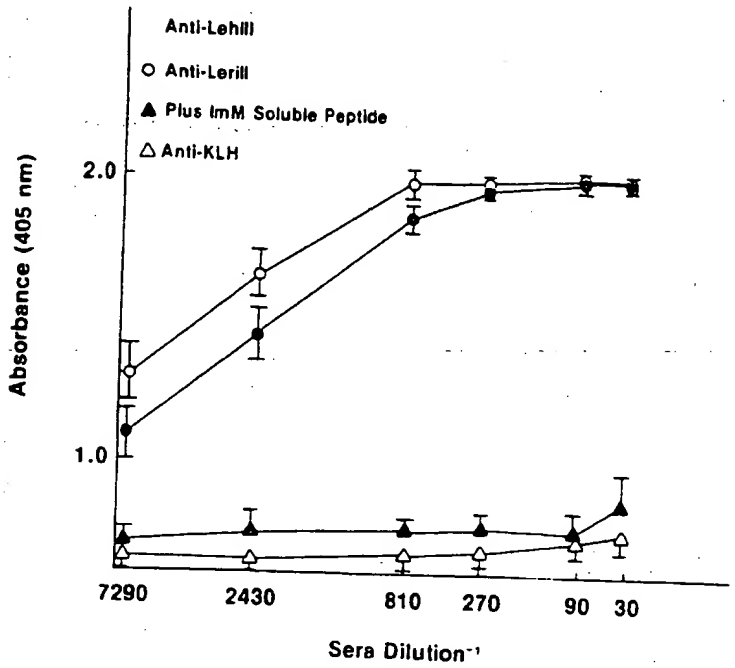


FIGURE 2

Reactivity of antisera against LEHLLL and LERILL on plates coated with LERILL. Sera from rats (3 per group) immunized with LEHLLL (●—●), LERILL (O—O) or KLH (Δ—Δ) were assayed for reactivity against LERILL using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM LEHLLL or 1 mM LERILL (▲—▲) to anti-LERILL sera during the reaction. Nonspecific binding is shown using anti-KLH sera. Each point represents the mean reactivity of three different rats ± standard deviations.

immune system as being almost identical. The ability of both antisera to recognize either peptide, and the ability to block both antisera reactivity with either peptide confirms this observation.

Anti-LERILL and anti-LEHLLL Antibodies React with IL-2

Since antisera against LERILL crossreacts with the IL-2 peptide, we questioned whether intact IL-2 would also be recognized. To demonstrate that antibodies specific for the HIV envelope peptide could react with IL-2, affinity chromatography was used. Each peptide was coupled to agarose beads, and the appropriate antisera was passed over each column. Figure 3 demonstrates the efficiency of the chromatographic procedure. Each purified antibody reacted with peptide, whereas the pass-through antisera did not. We concluded, therefore, that the affinity

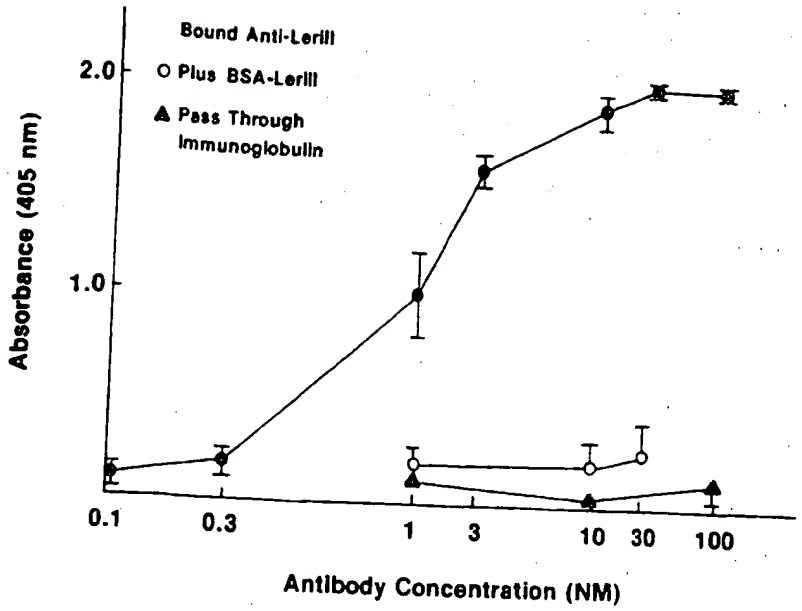


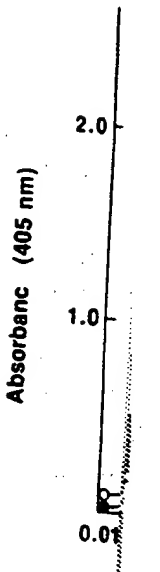
FIGURE 3

Reactivity of affinity purified anti-LERILL with LERILL. Sera from rats immunized with LERILL were passed over an agarose affinity column conjugated with LERILL. Antibody binding the column (●—●) and immunoglobulin which passed through the column (▲—▲) were assayed for reactivity against LERILL using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM soluble LERILL (O—O) to reactions involving antibody which bound to the column. Results are expressed as the mean of triplicate determinations ± standard deviations.

chromatography removed only the anti-peptide reactivity, since greater than 95% of the binding could be blocked with soluble peptide.

When IL-2 was coated onto plates, both anti-peptide antibodies reacted (see Figure 4). In fact, there was no significant difference between the ability of anti-LERILL or anti-LEHLLL antibodies to recognize IL-2. As little as 50 nM of either antibody could recognize IL-2. Thus, there is no doubt that specifically purified antibodies against the HIV envelope peptide, LERILL, react against IL-2.

If the IL-2 peptide, LEHLLL, is a significant epitope, then at least a portion of the antibodies raised against intact IL-2 should react with LEHLLL or its homologue, LERILL. To test this possibility, a rabbit anti-IL-2 antibody (Genzyme, Boston, MA) was allowed to react on IL-2 coated plates in the presence or absence of soluble peptide. Figure 5 demonstrates that either peptide could block approximately 25% of the heterologous antibody's binding. Considering that the epitope LEHLLL constitutes approximately 5% of the 15,500 dalton molecule weight of IL-2,



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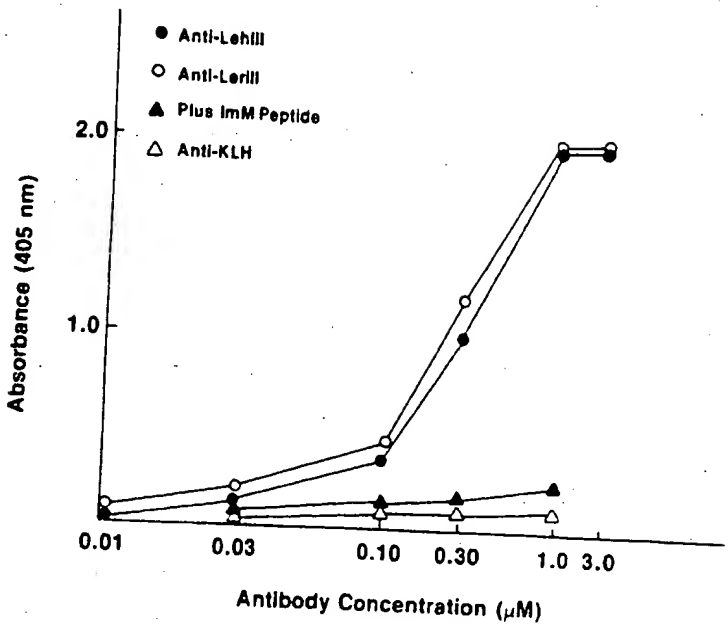

FIGURE 4

Reactivity of affinity purified anti-LEHLLL and anti-LERILL with IL-2. Affinity purified anti-LEHLLL (●—●), anti-LERILL (O—O), or anti-KLH (Δ—Δ) antibodies were assayed for reactivity against IL-2 using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM LEHLLL or 1 mM LERILL (▲—▲). Results are plotted as means of triplicate determinations. Standard deviations were never more than ± 10% of the mean experimental value.

the percentage of antibodies reacting with this epitope is disproportionately high relative to its contribution to the molecular weight. Thus, LEHLLL would appear to be a major epitope on the IL-2 molecule. More importantly, the ability of the HIV envelope peptide LERILL to block anti-IL-2 antibody reactivity with IL-2 again supports the concept of this peptide being a homologue of LEHLLL.

DISCUSSION

We show here that antibodies raised against an HIV envelope peptide, LERILL, crossreact with human IL-2. The basis for this crossreactivity is the similarity of the HIV envelope peptide with its IL-2 homologue, LEHLLL. Not only does antisera against LERILL recognize its IL-2 homologue, LEHLLL, and vice versa, but either soluble peptide could block this binding (Figures 1 and 2). Anti-peptide antibody binding directly to IL-2 could be

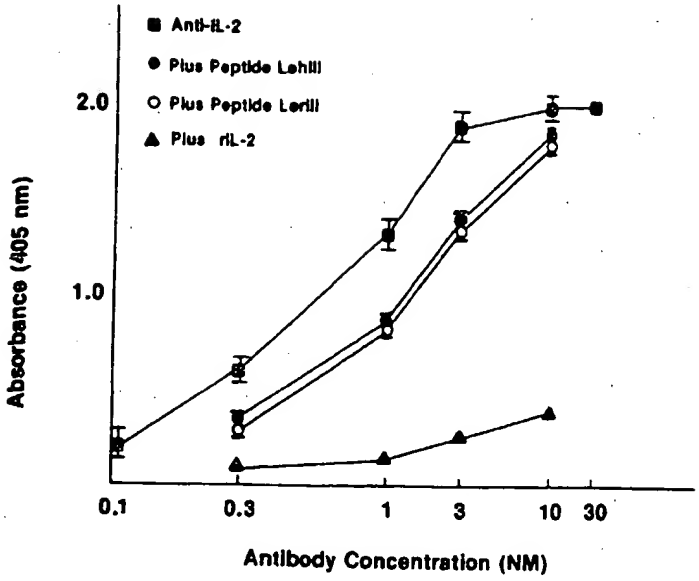

FIGURE 5

Inhibition of polyclonal anti-IL-2 binding by excess soluble LEHLLL or LERILL. Binding of a polyclonal anti-IL-2 antibody (■—■) was inhibited by 1 mM LEHLLL (●—●), 1 mM LERILL (O—O), or 1 μg per well recombinant IL-2 (▲—▲). Results are expressed as means of triplicate determinations ± standard deviations.

blocked by either soluble peptide as well (Figure 4). Thus, there can be no doubt that these two peptides represent crossreactive epitopes.

Establishing that the immune system recognizes these two peptide epitopes as being similar suggests a novel mechanism by which infection with HIV may contribute to the immunosuppression observed in this disease. Antibodies generated against IL-2 might be immunosuppressive since this lymphokine is of vital importance to immune competence (10). This possibility seems even more likely when one considers that the IL-2 sequence, LEHLLL, has been shown to be involved with IL-2 binding to the IL-2 receptor (1,12,13). If ongoing experiments in our laboratory do demonstrate that these crossreactive antibodies interfere with IL-2 mediated immune responses, then this would be a case where infection by a virus (i.e., HIV) results in the development of an autoimmune response (i.e., anti-IL-2 antibodies) by virtue of the presence of a crossreactive epitope present in the envelope protein.

In the majority of patients we have screened to date (15, manuscript in preparation), antibodies to the HIV envelope protein sequence, LERILL, have been detected. This finding may explain in part why serum IL-2 levels HIV-infected patients are low (16,17), and why IL-2 is needed to

reconstitute some in vitro responses using lymphocytes from HIV-infected patients (18-22). Future experiments will be directed at determining the extent at which these antibodies contribute to the immunosuppression which is a hallmark of AIDS.

ACKNOWLEDGEMENTS

Special thanks are given to Diane Weigent for preparing the manuscript and to Todd McBurnett for expert technical assistance. This work was supported by NIAID grant AI25078.

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Original Article

Possibilities of False Immunocytochemical Results Generated by The Use of Monoclonal Antibodies: The Example of the Anti-proinsulin Antibody[1]

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Received for publication December 19, 1994 and in revised form April 4, 1995; accepted April 26, 1995 (4A3559).

The immunocytochemical application of a monoclonal antibody (MAb) against the Arg-Arg region at the junction of the C-peptide and the insulin β-chain of the human proinsulin molecule on rat pancreatic tissue resulted in positive immunogold labeling over the insulin- as well as the glucagon-secreting cells. In the insulin cells, the Golgi cisternae and the immature clathrin-coated granules were intensely labeled, and in the glucagon cells all the granules were labeled. Control experiments using the anti-proinsulin MAb adsorbed with proinsulin, insulin, and glucagon have confirmed the crossreactivity of this proinsulin antibody with glucagon. Furthermore, the anti-proinsulin MAb appears to crossreact not only with rat but also with human glucagon and with bovine and porcine insulin and glucagon. Examination of the amino acid sequences of proinsulin and proglucagon has shown that both molecules display this Arg-Arg dipeptide sequence, which could explain the labeling obtained on both cell types. In addition, other propeptides also display such a sequence and, indeed, positive cells were detected in rat pituitary. These results demonstrate that by immunocytochemistry this MAb, although providing very specific results, reveals non-related molecules. This is due to the fact that these molecules exhibit similar short determinants. MAbs raised against very short amino acid sequences can reveal this determinant in various molecules, generating false-positive results. (*J Histochem Cytochem 43:881–886, 1995*)

KEY WORDS: Immunocytochemistry; Monoclonal antibodies; Insulin; Glucagon.

Introduction

The specificity of immunocytochemical results obtained at both the light and the electron microscopic level relies entirely on the properties of the first antibody, independent of the approach employed for the detection. Antibodies raised against a particular well-characterized antigen should be able to bind to this unique antigen and thus to discriminate it from all other proteins. This condition, which is a sine qua non for generating valuable results, has been somewhat challenged by the introduction of monoclonal antibodies (MAbs). Indeed, whereas polyclonal antibodies are composed of multiple species of immunoglobulins directed towards several determinants within a particular antigenic molecule, the MAbs are directed against single determinants consisting of very short sequences of amino acids (1). In this situation, although the MAbs are able to provide invaluable information about the molecular conformation of a particular determinant within that molecule, their use may in certain cases introduce some problems. In fact, although the specificity of the results is not affected, the multiplicity of molecules being recognized by a single MAb could be a problem. This is due to the existence of very similar determinants in various different peptides and proteins.

The aim of the present article is to report such a problem experienced with the use of a very well-characterized MAb directed against a very short determinant. Indeed, a particular MAb recognizing the region of the dipeptide Arg-Arg in the human proinsulin molecule (2) gives very specific and valid labeling in pancreatic insulin-secreting cells, revealing the sites of proinsulin molecules (3–5). However, because same or a similar determinant is also present in other peptides (6,7), the MAb generates positive labeling in other hormone-secreting cells, revealing not proinsulin but rather other non-related peptides. Concern is raised on this potential problem of getting false-positive cytochemical results by the use of certain MAbs.

Materials and Methods

For light microscopy, normal rat pancreatic tissue was fixed in Bouin's fixative and embedded in paraffin. For electron microscopy, human, rat, porcine, and bovine pancreatic tissues, as well as rat pituitary, were fixed by immersion with 1% glutaraldehyde and processed for embedding in Lowicryl K4M at −20°C (8). Paraffin sections were mounted on glass slides. Thin

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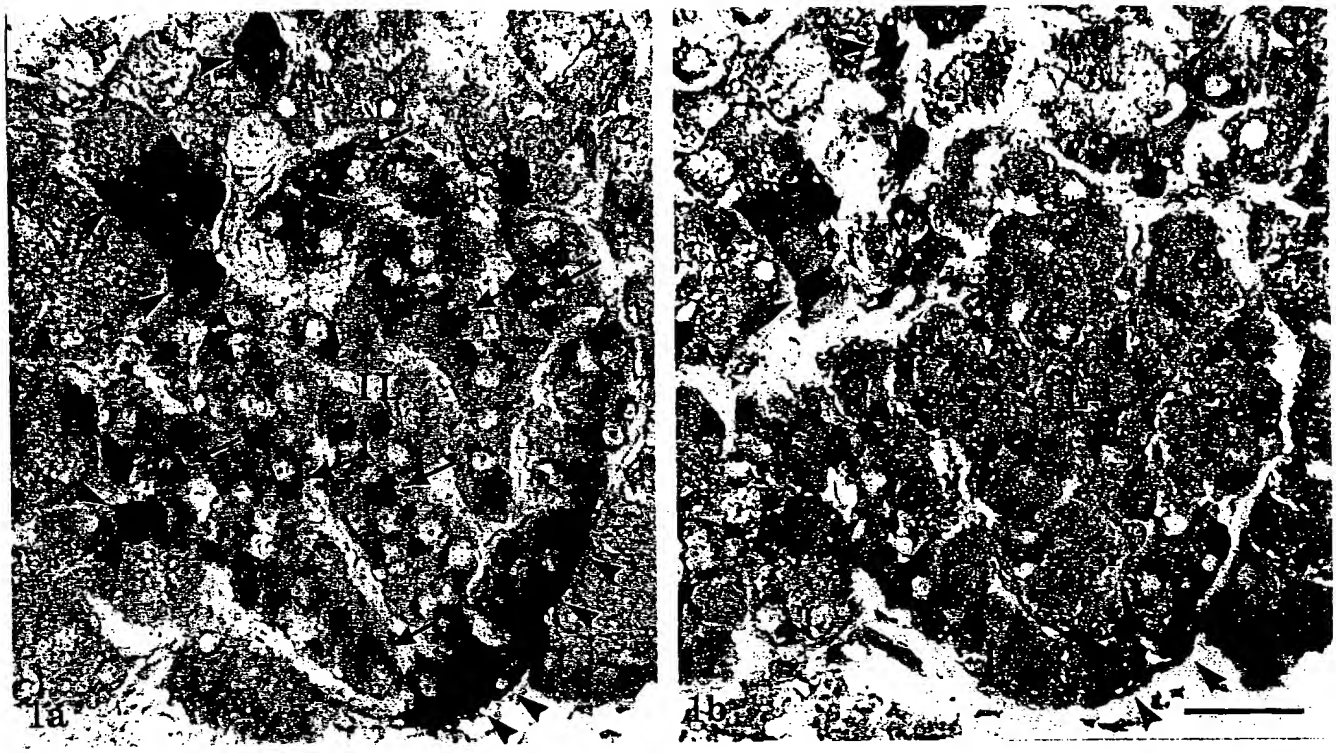


Figure 1. Light microscopic immunostaining on serial sections of an islet of Langerhans (IL) in rat pancreas. (a) Staining obtained with the anti-proinsulin MAb. (b) Staining obtained with the anti-glucagon antibody on a serial section. The anti-proinsulin MAb yields staining in the Golgi region (arrows) of the insulin-secreting cells in the central part of the islet. It also yields strong cytoplasmic staining in peripheral cells of the islets (arrowheads). On application of the anti-glucagon on a serial section (b), these same peripheral cells (arrowheads) display strong labeling. No staining was found on insulin-secreting cells with the anti-glucagon. Bar = 100 μm.

sections were mounted on nickel grids and processed for immunocytochemistry using a specific MAb and the protein A/G–gold or IgG–gold complexes.

The anti-human proinsulin MAb (Novo Nordisk BioLabs; Bagsvaerd, Denmark) used is directed against the region of the dipeptide Arg-Arg present at the junction between the C-peptide and the insulin β-chain within the human proinsulin molecule (2,9). It has been characterized by immunochemical and immunocytochemical techniques, having been used for specific demonstration of proinsulin molecules in rat pancreatic insulin-secreting cells (3,4). The anti-glucagon was a rabbit antiserum (INC; Stillwater, MN). The protein A/G–gold complex was prepared as reported previously with 10-nm gold particles (10); the IgG–gold complex was obtained from British BioCell (Cardiff, UK).

For light microscopic immunostaining, the sections were deparaffinized, rehydrated, and incubated with the MAb (dilution 1:10) for 2 hr at room temperature (RT). After rinsing with 0.01 M PBS, they were incubated with a secondary antibody, an anti-mouse IgG tagged with 10-nm gold particles, for 30 min at RT. The sections were further processed for the silver enhancement protocol (11) using the IntenSE M Silver Enhancement kit (Amersham Life Science; Poole, UK).

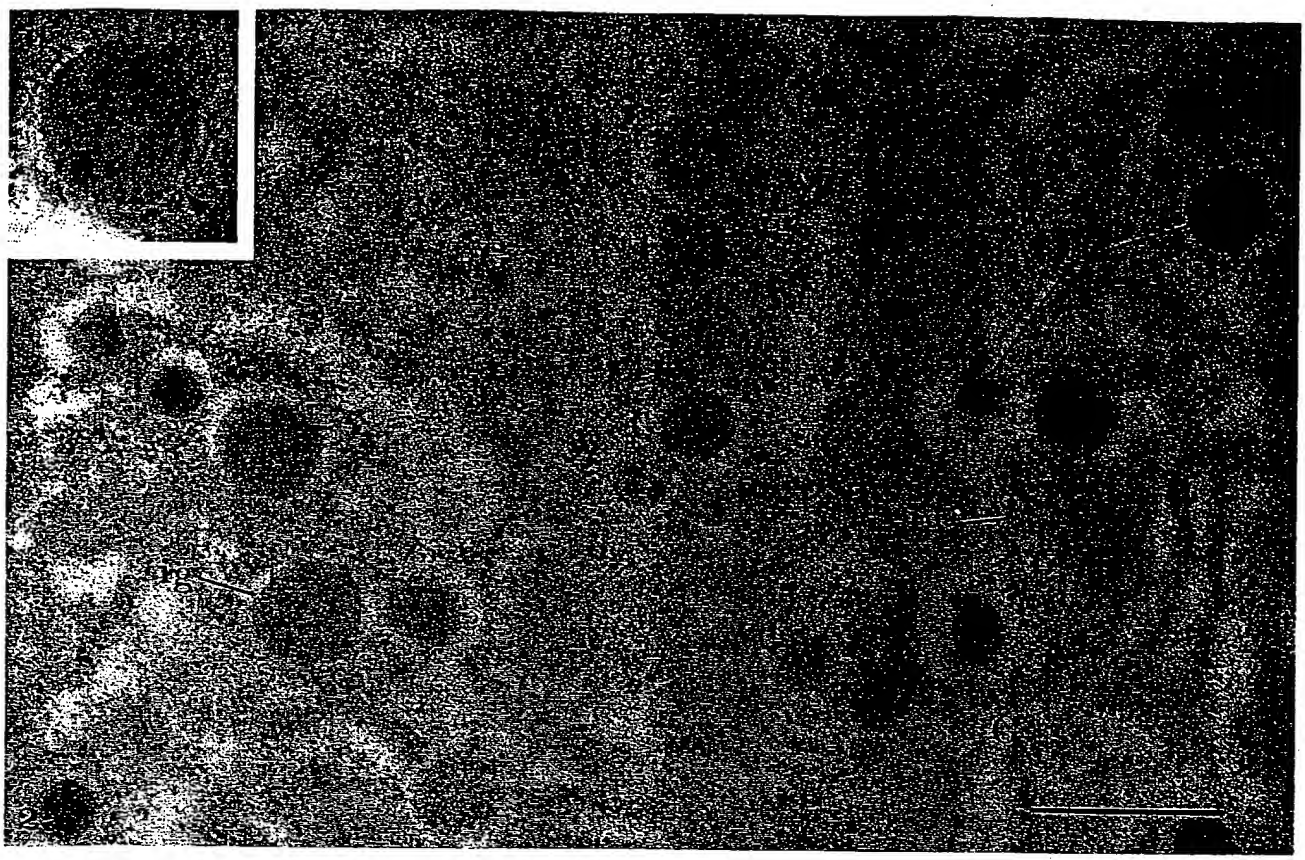
For electron microscopic immunolabeling, the thin sections were incubated with the same MAb (1:10 dilution) overnight at 4°C, followed by the protein A/G–gold complex for 30 min at RT. Sections were stained with uranyl acetate before examination with a Philips 410SL electron microscope.

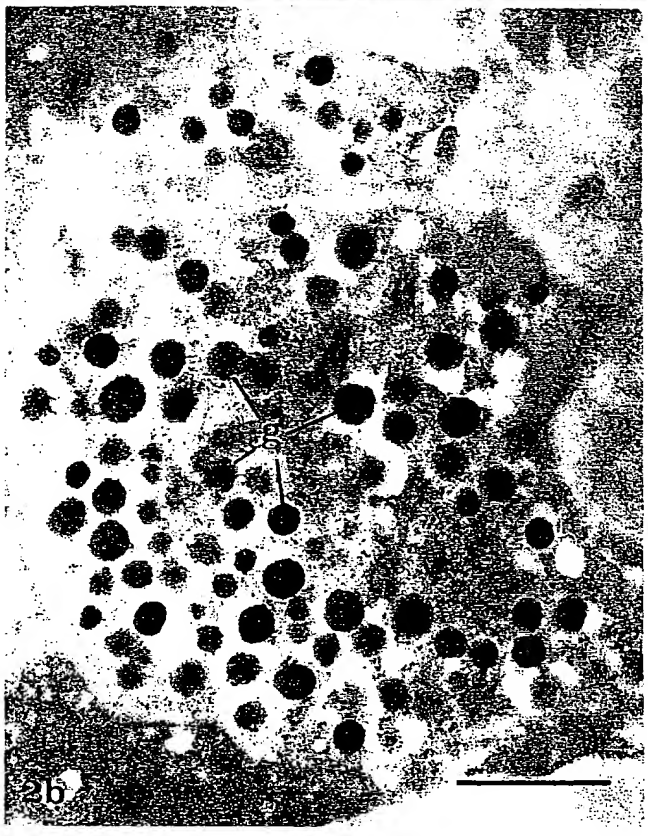
Control experiments were performed. Adsorption of the proinsulin MAb with different antigens was carried out before performance of the immunolabeling. Anti-proinsulin was adsorbed with insulin, proinsulin, and glucagon. The tissue sections were incubated with the adsorbed antibody and the protein A/G–gold complex according to the protocol described above.

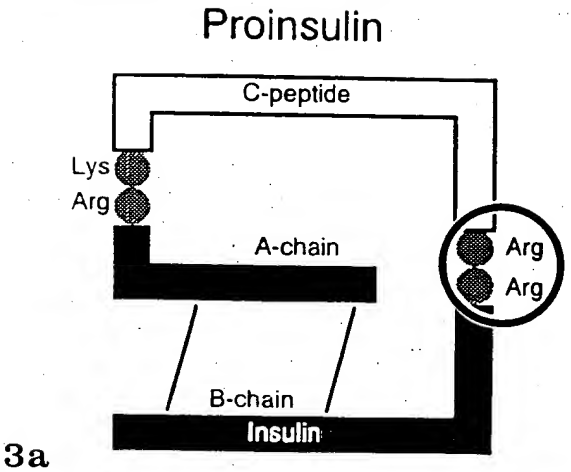
Results

By light microscopy, on application of the MAb against proinsulin, different cells of the islet of Langerhans in rat pancreas appeared labeled (Figure 1a). For the insulin-secreting cells located in the central part of the islet, the staining was not very intense but was concentrated in the Golgi area close to the nuclei. In contrast to this Golgi staining and in addition to the labeling of the insulin-secreting cells, other islet cells, few in number and located at the periphery of the islet, were also labeled and displayed very intense labeling involving the entire cell cytoplasm (Figure 1a). On applying the anti-glucagon antibody on a serial section, these same peripheral cells displayed positive labeling, demonstrating that they are in fact glucagon in nature (Figure 1b).

By electron microscopy on rat pancreatic tissue, the anti-proinsulin MAb yielded very specific labeling over the Golgi cisternae and over the light-cored immature secretory granules of the insulin-secreting cells (Figure 2a). In contrast, the mature insulin-containing granules, having a dense core, were not labeled. In several instances the labeled immature granules appeared delineated by a clathrin-coated membrane (Figure 2) as reported previously (3,4). In addition to labeling of the insulin-secreting cells, the







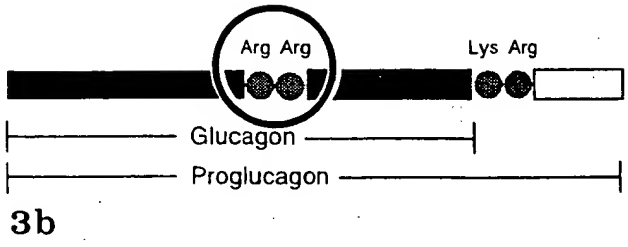


Figure 2. Electron microscopic immunolabeling obtained with the anti-proinsulin MAb on rat pancreatic tissue. (**a**) In insulin-secreting cells, labeling is particula intense over the Golgi apparatus (G) and the immature secretory granules (ig). The mature granules (g) appear free of labeling. (**Inset**) A labeled immature grani (ig) displaying the typical clathrin-coated membrane (arrow). (**b**) A glucagon-secreting cell labeled with the same anti-proinsulin MAb, displaying strong labeli over the glucagon secretory granules (g). Bars: **a** = 0.5 μm; **b** = 1 μm.

Figure 3. The molecular conformation of (**a**) proinsulin and (**b**) proglucagon, showing that both molecules possess the dipeptide Arg-Arg.

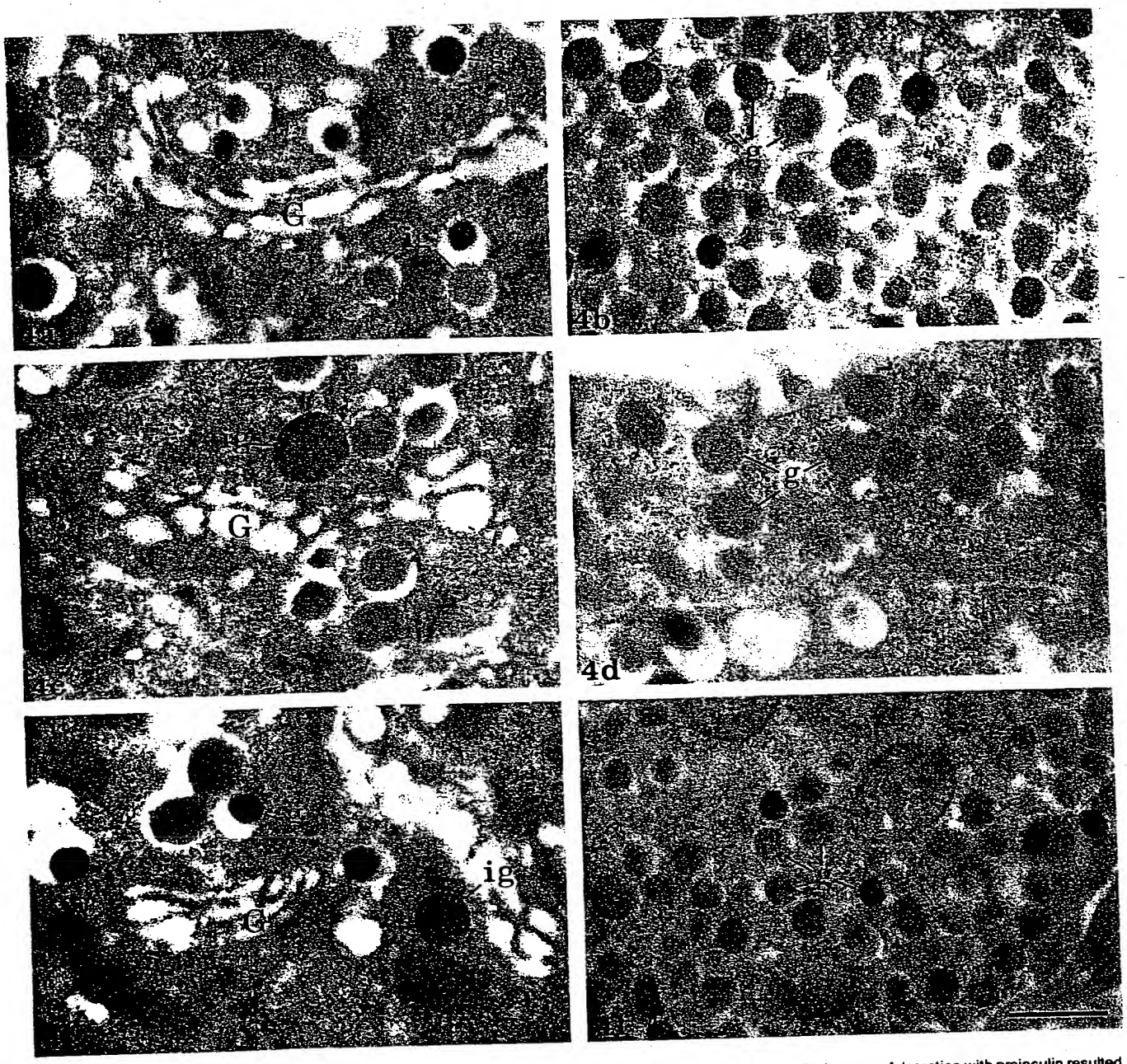


Figure 4. Control experiments. The anti-proinsulin antibody was adsorbed with (a,b) proinsulin, (c,d) insulin, and (e,f) glucagon. Adsorption with proinsulin resulted in complete elimination of the specific labeling in both the insulin (a) and the glucagon cells (b), whereas adsorption with insulin did not affect the intensity of labeling (c,d). For adsorption with glucagon, the intensity of labeling in insulin cells (e) was reduced, and that in glucagon cells was almost abolished (f). G, Golgi apparatus; ig, immature secretory granules; g, secretory granules. Bars = 0.5 μm.

glucagon-secreting cells, characterized by their dark granules having a very narrow clear halo and their peripheral location in the islet, were also labeled, displaying a significant number of gold particles over their secretory granules (Figure 2b). Somatostatin cells remained unlabeled (not shown).

Examination of the amino acid sequences of proinsulin and proglucagon molecules reveals similarities, with the existence of the dipeptide Arg-Arg in both molecules (Figure 3).

Control experiments using adsorbed anti-proinsulin were carried out with different antigens. When the anti-proinsulin was adsorbed with proinsulin, a drastic decrease in labeling intensity was observed in the immature secretory granules of the insulin cells, as well as in the glucagon secretory granules (Figures 4a and 4b). When it was adsorbed with insulin, no variation in the labeling pattern was detected in either type of cell (Figures 4c and 4d). Indeed, the immature secretory granules of the insulin cells and all the secretory granules of the glucagon cells remained intensely labeled. When the antibody was adsorbed with glucagon, a signifi-

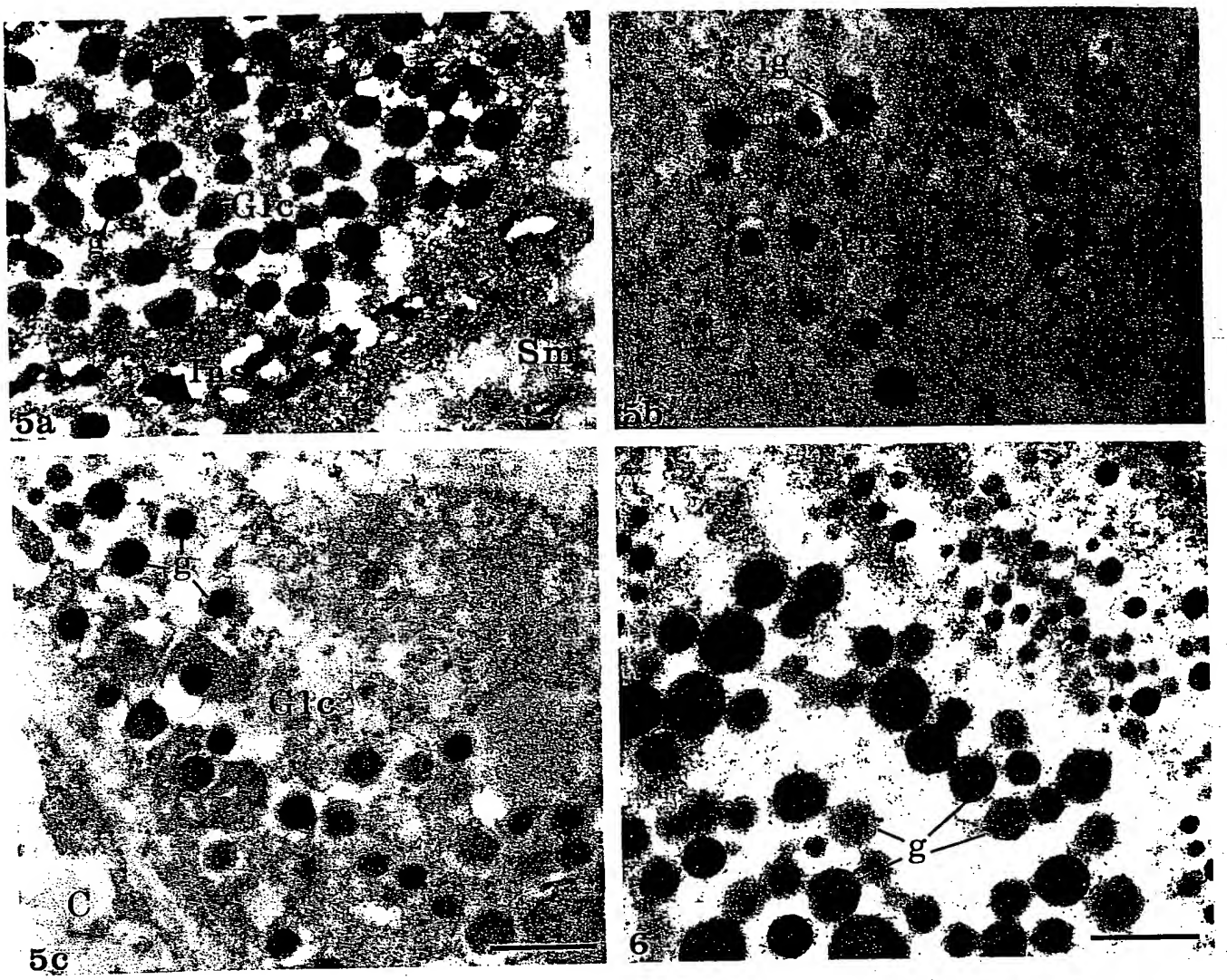


Figure 5. Immunolabeling obtained on (a) human, (b) bovine, (c) porcine insulin or glucagon cells with the proinsulin antibody. Most of the glucagon secretory granules in human and porcine cells (a,c), as well as some insulin secretory granules in bovine cells, probably immature ones (b), appear labeled by gold particles. Glc, glucagon cell; Ins, insulin cell; Sm, somatostatin cell; ig, immature granules; g, secretory granules; C, blood capillary. Bar = 0.5 μm.

Figure 6. Immunolabeling obtained on rat pituitary tissue with the proinsulin antibody. Some cells, characterized by their large dense granules (g), appear labeled by gold particles, whereas neighboring cells displaying small granules are devoid of labeling. Bar = 0.5 μm.

cant decrease in labeling was noted in the immature secretory granules of the insulin-secreting cells (Figure 4e), and the glucagon secretory granules were almost free of any labeling (Figure 4f).

Application of the anti-proinsulin antibody on human, porcine, and bovine pancreatic tissues resulted in specific immunolabeling on insulin- and on glucagon-secreting cells (Figure 5). For the insulin-secreting cells, labeling was restricted to a few secretory granules, which we assumed to be immature. The poor ultrastructural preservation, particularly on human tissue, did not permit identification of the clathrin coating of these granules. On the other hand, in glucagon-secreting cells most of the granules were labeled with the anti-proinsulin MAb, confirming the results obtained on the rat tissue (Figure 5). Somatostatin cells remained negative (Figure 5a).

Application of the anti-proinsulin MAb on tissue sections of rat pituitary revealed immunogold labeling over some endocrine cells (Figure 6). These cells were characterized by numerous large dark granules which displayed intense labeling. Neighboring cells containing small light granules were devoid of labeling (Figure 6).

Discussion

In the present study we used a very specific and well-characterized MAb raised against a very narrow and definite region in the human proinsulin molecule at the junction between the C-peptide and the insulin β-chain. This area comprises a particular dipeptide, Arg-Arg, which is the target of the convertase PC1 and which participates in the conversion of the proinsulin into C-peptide and

insulin (4,7,12). The MAb has been well characterized (2,9) and has been used in different studies for detection of proinsulin molecules within insulin-secreting cells (3–5). Indeed, confirming previous reports (3,4), application of this MAb with the immunogold approach on insulin-secreting cells led to labeling restricted to the Golgi cisternae and the immature clathrin-coated secretory granules (Figure 2). However, this same MAb gave a very defined and specific labeling over the glucagon-secreting cells, particularly at the level of the secretory granules. Analysis of the amino acid sequence of proinsulin and proglucagon molecules shows that the dipeptide region Arg-Arg is in fact present within both prohormone molecules (Figure 3). The presence of this determinant in the glucagon molecule could therefore explain the specific and strong labeling found in the glucagon-containing granules, as well as in the results obtained by the control experiments.

By immunochemical techniques, this proinsulin MAb was shown to recognize the Arg-Arg region of the human proinsulin molecule and not to cross-react with bovine or porcine proinsulin (2,9). However, by immunocytochemistry, this same MAb appears not only to react with human proinsulin but also to crossreact with porcine, bovine, and rat proinsulin. In addition, it also binds glucagon molecules in pancreatic cells from all of the above-mentioned species. Moreover, it also appears to recognize some rat pituitary hormones, probably the pro-opiomelanocortin, which displays similar Arg-Arg regions (6). Although no biochemical test of crossreactivity was performed with other prohormones, the immunochemical techniques have assigned a very narrow specificity to this antibody (2,9). The wider crossreactivity displayed by this antibody when used with morphological means could be based on the concept proposed (9) that the antibody recognizes not only a very particular amino acid sequence but also the three-dimensional conformation of this determinant. The processing of the tissues for morphological studies, which is known to affect the structure of proteins, may favor particular conformation(s) of similar determinants to resemble the domain present in the parent molecule.

Short amino acid sequences of identical nature can be present in various proteins, and an antibody directed against such a sequence, although still yielding specific labeling, could reveal different molecules not related to the original antigen. This problem, already discussed by Mason et al. (13) and illustrated by the present results, raises the possibility of false-positive labelings with monoclonal antibodies and stresses the care that should be taken before any rapid interpretation of immunocytochemical results.

Acknowledgments

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Effects of Amino Acid Substitutions Outside an Antigenic Site on Protein Binding to Monoclonal Antibodies of Predetermined Specificity Obtained by Peptide Immunization: Demonstration with Region 94–100 (Antigenic Site 3) of Myoglobin

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Amino acid substitutions outside protein antigenic sites are very frequently assumed to exert no effect on binding to antiprotein antibodies, especially if these are monoclonal antibodies (mAbs). In fact, a very popular method for localization of residues in protein antigenic sites is based on the interpretation that whenever a replacement causes a change in binding to antibody, then that residue will be located in the antigenic site. To test this assumption, mAbs of predetermined specificity were prepared by immunization with a free (i.e., without coupling to any carrier) synthetic peptide representing region 94–100 of sperm whale myoglobin (Mb). The cross-reactivities and relative affinities of three mAbs with eight Mb variants were studied. Five Mb variants which had no substitutions within the boundaries of the designed antigenic site exhibited remarkable, and in two cases almost complete, loss in cross-reactivity relative to the reference antigen, sperm whale Mb. Two myoglobins, each of which had one substitution within region 94–100, showed little or no reactivity with the three mAbs. It is concluded that substitutions outside an antigenic site can exert drastic effects on the reactivity of a protein with mAbs against the site and that caution should be exercised in interpreting cross-reactivity data of proteins to implicate residues directly in an antigenic site.

KEY WORDS: Amino acid substitutions; monoclonal antibodies; myoglobin; predetermined specificity; synthetic antigenic site.

1. INTRODUCTION[3]

Immunochemical cross-reactions of protein mutants are very often used to implicate the involvement of certain amino acid residues on a protein in its antigenic sites. The data are interpreted on the basis of the assumption that every amino acid replacement on the antigen which results in changes in its binding properties is directly involved in the interaction with antibody, particularly when a monoclonal antibody (mAb) is used (Berzofsky *et al.*, 1980, 1982, 1983; van Regenmortel, 1988). On the other hand, it has been shown that substitutions outside protein antigenic sites will influence the antigenic reactivity of the site because of one or more of the following factors: conformational and steric readjustments, electron delocalization, charge redistribution, and changes in hydrogen bonding or hydrophobic interactions (Kazim and Atassi, 1980; Atassi and Kazim, 1980;

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[3] Abbreviations used: BSA, bovine serum albumin; mAb, monoclonal antibody; mAb C_{50}, monoclonal antibody concentration at 50% cross-reactivity; Mb, myoglobin and when preceded by an abbreviation it denotes the following Species: Bg, badger, Ch, chicken; Cs, California sea lion; Dg, dog; Ed, echidna; Fb, finback whale; Pd; pacific common dolphin; Sp, Sperm whale; PBS, 0.15 M Nacl in 0.01 M sodium phosphate buffer, *p*H 7.2; RIA, radioimmune assay.

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Twining *et al.*, 1981). These latter studies were done with polyclonal antibodies whose recognition was directed at a number of antigenic sites in each multideterminant protein antigen [five in Mb (Atassi, 1975) and three in lysozyme (Atassi, 1987)]. Therefore, the assignment of the effects of substitutions on a given site was difficult to determine and could only be estimated from their distances in the three-dimensional (3D) structure from the site residues. Clearly, to resolve this issue, mAbs against a single site are needed whose specificity is known precisely and which is not deduced by the very approach it is set out to test.

To determine in an unequivocal manner whether the reactivity of an antigenic site is influenced by substitutions outside the site, we have employed here monoclonal antibodies that were generated by immunization with a synthetic peptide representing region 94–100 [antigenic site 3 (Atassi, 1975)] of SpMb. The peptide was used as an immunogen in its free state (i.e., without coupling to any carrier) to produce mAbs of predetermined specificity (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*; for review, see Atassi and Young, 1985; Atassi, 1986). Cross-reactions of Mbs which do not have substitutions in this region revealed that substitutions outside the site can exert dramatic effects on the binding of a protein with anti-site mAbs.

2. MATERIALS AND METHODS

2.1. Materials

The isolation and purification of myoglobins of various species were performed by previously reported procedures (Atassi, 1964, 1970; Twining *et al.*, 1980). BgMb, CsMb, and PdMb were obtained from Dr. C. R. Young (Texas A&M University). The homogeneity of each Mb was confirmed by polyacrylamide-gel electrophoresis. The synthetic peptide, Ala-Thr-Lys-His-Lys-Ile-Pro, corresponds to antigenic site 3 (residues 94–100) of SpMb (Atassi, 1975). Synthesis, purification, and characterization of the peptide have been described previously (Pai and Atassi, 1975). The synthetic peptide was used as an immunogen *in its form* (i.e., without coupling to any carrier) in Balb By J mice (6–8 weeks). The mice were injected and boosted biweekly with 25 μg of peptide [previously found (Young *et al.*, 1983) to be the optimum dose in this strain] as an emulsion of a solution in PBS (50 μl) with an equal volume of Freund's complete adjuvant. The mice received boosters until good antibody titers against the peptide and SpMb were obtained in the sera. Monoclonal antibodies were prepared as described (Schmitz *et al.*, 1982, 1983*a*, *b*). Sera and culture supernatants were screened for hybridoma antibodies by a solid-phase RIA on poly(vinylchloride) plates (Costar, Cambridge, Massachusetts) using the peptide-BSA conjugate and SpMb as the plate antigens (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*).

2.2. Determination of PVC-Plates Capacity for Various Myoglobins

It was necessary to rule out the possibility that any differences that might be found in the binding of mAbs to the various Mbs will not be caused by differences in the amounts of Mbs bound to the plates. Therefore, the amount of each Mb required to saturate the plate was determined. Each protein (25 μg) was labeled with ^{125}I by the chloramine-T method (Hunter, 1969). A fixed amount of each Mb (5×10^5 cpm) was mixed with the respective unlabeled Mb (25 μg). Serial dilutions were made and 50 μl aliquots of each dilution were plated in triplicate into the wells of the plate. The plates were incubated at 37°C for 3 hr, after which they were washed six times with PBS, dried, cut out, and counted in gamma counter. The amount of each labeled Mb bound (in cpm) was plotted against its concentration. From these plots, it was possible to determine, for each Mb, the concentration needed to saturate the well.

2.3. Determination of Cross-Reactivities and Relative Affinities

Cross-reactivity studies were done as described by East *et al.*, (1982) and Leach (1983). In the present work, pvc plates were incubated (37°C, 3 hr) with saturating amounts (50 μl of 50 μg/ml) of various myoglobins after which they were blocked (37°C, 1 hr) with 100 μl of 1% BSA in PBS. Aliquots (50 μl) of serial dilutions of each mAb (in 0.1% BSA in PBS) were added to the wells and the plates were incubated at room temperature for 5 hr and then washed (five times) with PBS. The second antibody (rabbit anti-mouse IgMk), appropriately diluted with PBS-0.1 BSA, was added (50 μl) and the plates were incubated at 37°C for 2 hr. The plates were washed six times with PBS and then ^{125}I-labeled protein A (2×10^5 cpm) was added in 50 μl of PBS-0.1% BSA. Following incubation at room temperatue for 2 h, the plates were

washed with PBS and dried. The wells were then cut out and their radioactivity measured in a gamma counter. Nonspecific binding was calculated from the binding of antipeptide mAbs (the test antibodies) to wells coated with BSA. Additional negative controls included the binding, to each test Mb, of normal mouse and human IgM and culture supernatant from P3 × 63-Ag8-653 myeloma cell lines. The results were expressed in percent cross-reactivity relative to antibody bound to SpMb as 100%. Measurements of relative binding affinities were determined by antibody dilution analysis (van Heyningen, 1986). Serial dilutions of mAb were allowed to bind to each Mb as described above. Affinities were ranked on the basis of the concentration of antibody required to give 50% of maximum binding (C_{50}) relative to the C_{50} with SpMb as 1.00. This relative ranking of affinities is valid only for mAbs which bind to a single determinant on the antigen used for dilution analysis, which is the case here. Concentrations of mAb solutions were determined by calibrated solid-phase RIA relative to standard curves of known concentrations of affinity-purified mouse IgMk.

2.4. Calculation of the Distance between Site Residues and All the Other Mb Residues

The C^{α}-to-C^{α} distances as well as the atoms of nearest approach between each of the residues 94 through 100 (antigenic site 3) of SpMb and all other residues in the molecule were calculated, from the SpMb coordinates at 2.0′ Å (Takano, 1984), on a VAX 8550 using the program chain (Sacks, 1988). These distances had previously been calculated (Kazim and Atassi, 1980) but subsequent refinement of Mb structure (Takano, 1984) necessitated the recalculation of these distances.

3. RESULTS

3.1. Binding of the Myoglobins to the Plates

It was necessary to ensure at the outset that the differences in the antigenic reactivities of the various Mbs were not caused by differences in the amounts of each Mb bound to the plates. The results (Fig. 1) using ^{125}I-labeled Mbs showed that each Mb was able to saturate the well when its concentration was 25 μg/ml (1.5 μg/well). Therefore, in all subsequent studies, the Mb solutions employed 50μg/ml (2.5 μg/well), which is twice the concentration necessary to saturate the wells.

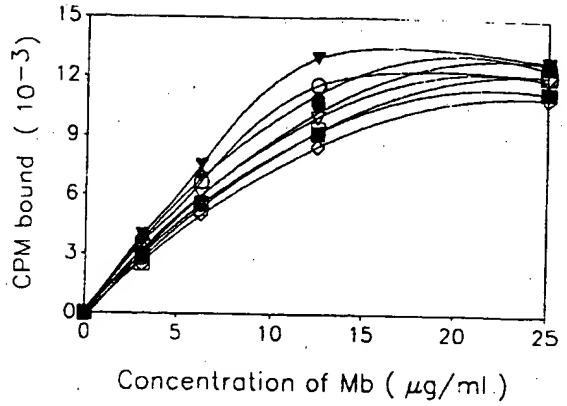

Fig. 1. Determination of PVC-plate binding capacity to myoglobin variants. The binding capacity of the wells of PVC plates for the various myoglobins was determined using increased amounts of ^{125}I-labeled Mb and unlabeled Mb: (○) BgMb, (■) ChMb, (◆) CsMb, (▼) DgMb, (▽) EdMb, (◇) FbMb, (□) PdMb, and (●) SpMb. For experimental details, see the text.

3.2. Characterization of Antipeptide Antibodies

Pre-immune sera from the mice showed no antibody binding to the immunizing peptide 94–100 or to SpMb. The mice were injected with the optimum dose (25 μg/mouse) of peptide 94-100 and boosted until the sera possessed high titers of antibodies that bound to the peptide and to SpMb. The polyclonal antisera and the mAbs obtained from the mouse B cells after hybridization were entirely specific to the peptide and SpMb. They did not react with any SpMb peptides or any other proteins and peptides (from our large library of synthetic peptides) that are not related to the Mb system. The specificity of peptide-generated mAbs was in agreement with previous reports (Schmitz *et al.*, 1983*a*, *b*; Atassi and Young, 1985).

A panel of 18 mAbs giving high levels of binding to SpMb was prepared from fusion of spleen cells (6 mAbs) and of lymph node cells (12 mAbs). All the mAbs (as well as the polyclonal antisera) were of IgM class and *k* light chain. Three mAbs (nos. 708, 718, and 720) were used for this work on the basis of their higher relative-binding affinities

3.3. Binding of the Monoclonal Antibodies to the Various Myoglobins

Of the myoglobins prepared for those studies, five have no substitutions within the region 94–100 (to which the antibody specificity is directed) and two have each one substitution in the site (Table I).

Table I. Antigenic Site 3 of Sperm Whale Mb and Substitutions Within This Region in Other Myoglobins[a]

Myoglobin	Residue no.	94	Site 3 95	96	97	98	99	100
Sperm whale		Ala	Thr	Lys	His	Lys	Ile	Pro
Finback whale								
Pacific common dolphin								
California sea lion								
Dog								
Chicken								
Echidna								Ser
Badger							Val	

[a] The amino acid substitutions were based on the sequences given in the references cited: sperm whale Mb (Edmundson, 1965; Romero-Herrera and Lehmann, 1974); finback whale Mb (DiMarchi *et al.*, 1978); Pacific common dolphin Mb (Wang *et al.*, 1977); California sea lion Mb (Vigna *et al.*, 1974); dog Mb (Dumur *et al.*, 1976); chicken Mb (DeConinck *et al.*, 1975; Romero-Herrera *et al.*, 1978); echidna Mb (Castillo *et al.*, 1978), and badger Mb (Tetaert *et al.*, 1974).

The myoglobins that have no substitutions within region 94–100 are CsMb, FbMb, PdMb, ChMb, and DgMb. The cross-reactions of these myoglobins with antipeptide 94–100 mAbs 708, 718, and 720 were determined as a function of mAbs concentration. The results of binding to CsMb, FbMb, and PdMb are shown in Fig. 2, while Fig. 3 summarizes the results with ChMb and DgMb. With a given mAb, the cross-reactivity of each of these five myoglobins was markedly decreased relative to the reference antigen, SpMb. The extents of maximum cross-reactions (relative to SpMb as 100%) of CsMb, FbMb, and PdMb with mAbs 708, 718, and 720 in that order were CsMb, 47%, 35%, and 43%; FbMb, 47%, 37%, and 64%; PdMb, 39%, 40%, and 61%. In the case of ChMb and DgMb, their cross-reactions were dramatically and unexpectedly low (less than 20%, Fig. 3).

For the two myoglobins of eschidna and badger, each has one substitution within region 94–100 (Table I). These two myoglobins showed little or no reactivity with the three mAbs (Fig. 4).

Expression of the results in terms of the relative-binding affinities (Fig. 5) showed that all seven myoglobins (including the five that have no substitutions within the boundaries of the site) had greatly reduced binding affinities, relative to SpMb. With only one mAb (no. 720), two myoglobins that have no substitutions in the site (PdMb and FbMb) showed relative affinities that were measurable by this method, and even these were much lower (70%) than that of SpMb with this mAb.

4. DISCUSSION

It was found relatively early that the reaction of a protein antigen with its antibodies is influenced by conformational changes (Atassi, 1967, 1970; Habeeb and Atassi, 1971). The first clear evidence of this was in fact obtained with Mb and hemoglobin. In these two proteins, even though the heme group is not part of an antigenic site (Reichlin *et al.*, 1963; Atassi, 1967), derivatives whose conformation was intentionally altered using hemes with modified side chains or metal substitutions (to replace the iron) exhibited remarkable changes in antigenic reactivity (Atassi, 1967; Atassi and Skalski, 1969; Andres and Atassi, 1970). Subsequent findings with other protein systems (Atassi *et al.*, 1970; Habeeb and Atassi, 1971; Arnon and Maron, 1971, Arnheim, 1973; Prager *et al.*, 1974; Atassi and Habeeb, 1977; Habeeb, 1977) made it evident that the antibody response against native protein antigens is directed against their native three-dimensional structure (Atassi, 1967, 1975, 1978, 1984). This is now a well-established principle in molecular immunology.

Amino acid substitutions can cause conformational readjustments which will influence antigenic reactivity (Atassi, 1970; Atassi *et al.*, 1970; Arnheim, 1973, Prager *et al.*, 1974). Quite often, however, amino acid substitutions do not induce any detectable conformational changes but will nevertheless influence antigenic reactivity. The replacements may result in electron delocalization and charge redistribution and/or changes of hydrogen bonding and hydrophobic interactions which could alter the binding properties of an antigenic site. Indeed, studies with Mb (Kazim and Atassi, 1980; Twining *et al.*, 1980) and lysozyme (Atassi and Kazim, 1980) showed that substitutions outside the antigenic sites of the respective protein (Atassi, 1975, 1978) exerted remarkable effects on its antigenic reactivity, and it was concluded that not every replacement that causes a change in

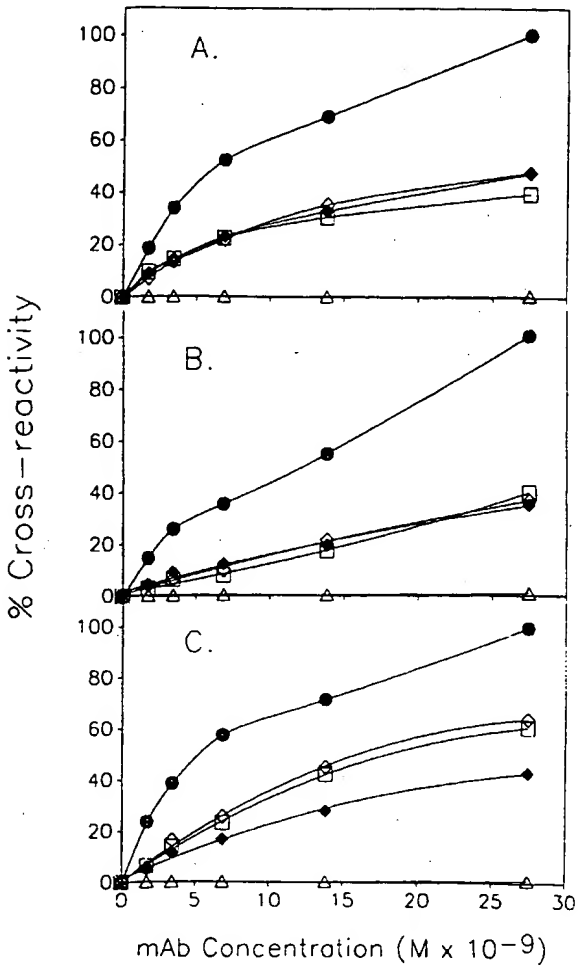

Fig. 2. Cross-reactivity of antisite 3 mAbs with PDMb, FbMb, and CsMb, which have no substitutions in this site. This binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentration, to SpMb (●), PdMb (□), FbMb (◇), and CsMb (◆) was determined. The microtiter wells were coated with 50 μl of Mb solutions having the same protein concentration (50 μg/ml) and the assays were done as described under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to the maximum bound by SpMb as 100%) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, PdMb, FbMb, and CsMb.

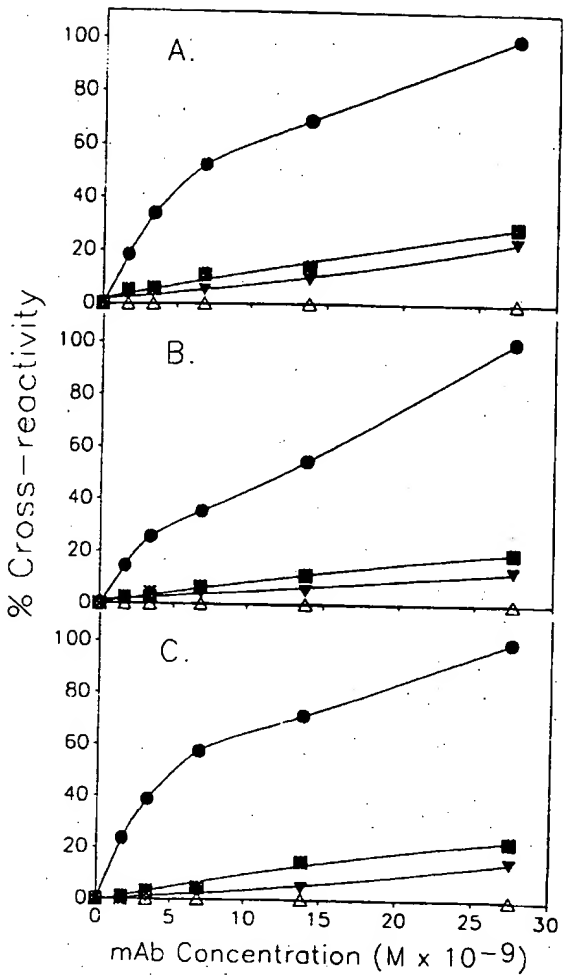

Fig. 3. Cross-reactivity of antisite 3 mAbs with ChMb and DgMb which have no substitution in that site. The binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentration, to SpMb (●), ChMb (■), and DgMb (▼) was determined. The microtiter wells were coated with (50 μl of Mb solutions having the same antigen concentration (50 μg/ml)). The assays were done as described under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to the maximum bound by SpMb) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, ChMb, and DgMb.

antigenic reactivity will necessarily reside in an antigenic site.

On the other hand, others have preferred to ascribe a direct role for all replacements that influence reaction of antigen with antibody, particularly when the latter is a monoclonal antibody. Thus, it has been clearly stated that "amino acid substitutions which affect binding by monoclonal antibodies are most likely to be within the antigenic site than far from it" (Berzofsky *et al.*, 1982). More recently, it was similarly

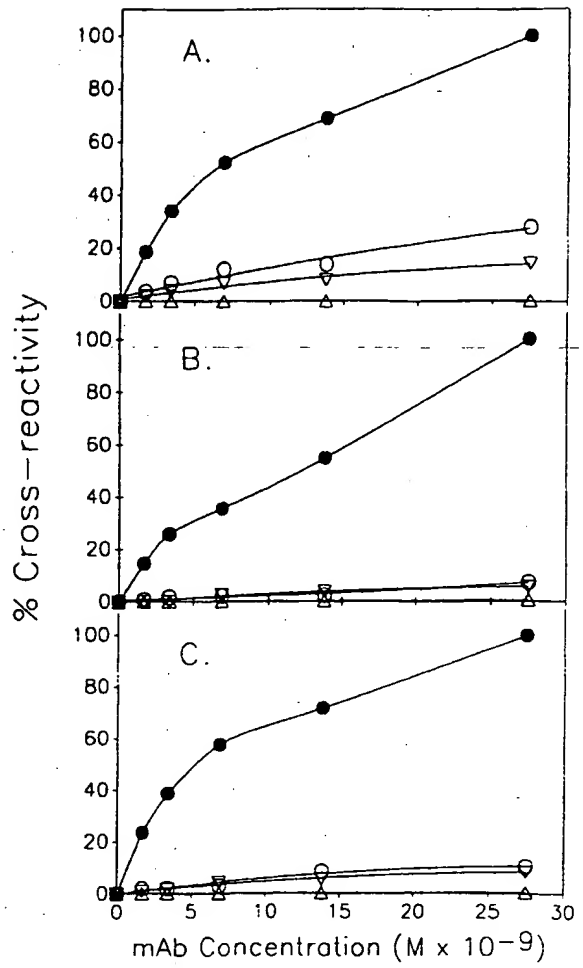

Fig. 4. Cross-reactivity of antisite 3 mAbs with EdMb and BgMb which have substitutions in and outside the site. The binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentrations to microtiter wells coated with 50 μl of SpMb (●), EdMb (▽), and BgMb (○) having the same antigen concentration (50 μg/ml) was done as shown under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to maximum bound by SpMb as 100%) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, EdMb, and DgMb.

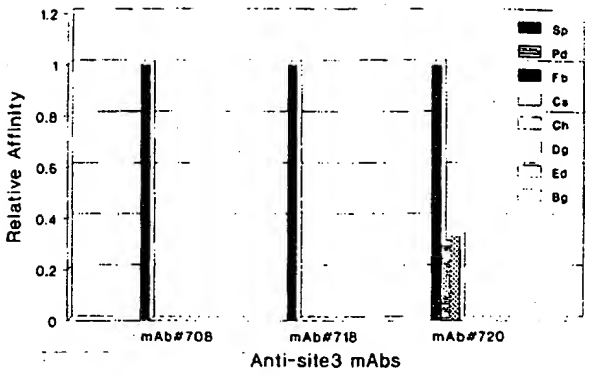

Fig. 5. Relative binding affinities of antisite 3 mAbs to myoglobin variants. The relative binding of antisite 3 mAbs to each myoglobin is calculated from mAb concentrations at 50% cross-reactivity relative to SpMb "the reference antigen" as 1.0. The relative binding affinities of mAbs 708 and 718 (to PdMb, FbMb, CsMb, ChMb, DgMb, EdMb, and BgMb) and of mAb 720 (to CsMb, ChMb, DgMb, and BgMb) could not be obtained because their maximum cross-reactivities were <50% (see Figs. 2–4).

stated that "if the substitution leads to a change in antibody binding, the mutated residue is likely to be directly involved in the structure of an epitope". And this method gives unambiguous information only when monoclonal antibodies are used as probes and the tertiary structure of the protein is known" (van Regenmortel, 1988). Based on this method of interpretation, a large number of protein antigenic sites have been described (e.g., Reichlin, 1972; Urbanski and Margoliash, 1977; Reichlin and Eng, 1978; Berzofsky *et al.*, 1982; Benjamin *et al.*, 1984; Hannum and Margoliash, 1985; Berzofsky and Berkower, 1989).

The findings mentioned above—which showed (Kazim and Atassi, 1980; Atassi and Kazim, 1980; Twining *et al.*, 1980) that substitutions outside the known antigenic sites of Mb (Atassi, 1975) and lysozyme (Atassi, 1978) influenced the antigenic reactivity of each protein with its respective antibodies—were done with polyclonal antiprotein antibodies. The specificities of these antibodies had been mapped exhaustively by a number of independent chemical, synthetic, and immunochemical approaches (Atassi, 1975, 1978). Because the polyclonal antibodies were capable of binding to a number of antigenic sites (five in Mb and three in lysozyme), the effects of substitutions on the binding properties of a given antigenic site were difficult to isolate and were estimated from the distances of the replacements from site residue (Kazim and Atassi, 1980; Twining *et al.*, 1980). A simpler system was therefore required to examine in an unequivocal manner the effect of substitutions outside an antigenic site whose boundaries are known precisely. This would also resolve whether cross-reactivities of protein mutants is a reliable approach for localization of antigenic sites. Clearly, to investigate

Table II. Nearest-Neighbour Residues to Antigenic Site 3 of Sperm Whale Mb[a]

Nearest neighbor residue	Minimum distances and closest atoms to residues in antigenic site 3						
	Ala-94	Thr-95	Lys-96	His-97	Lys-98	Ile-99	Pro-100
Lys-42					4.43 NZ–O	5.29 CG–CD_1	4.48 CE–CD
Phe-43						6.53 CE_2–CD_1	
Ala-90	5.70 O–N						
Ser-92			6.94 O–N	4.22 O–N			
His-93				4.80 O–C		5.75 O–C	
Ala-94	C–N	3.95					
Thr-95	3.94 N–C						
Lys-96				6.66 C–N			
His-97			6.66 N–C			6.79 C–N	
Ile-99				6.79 N–C			5.04 C–N
Pro-100						5.04 N–N	
Tyr-103							4.92 N–O
Leu-104						6.89 CD_2–CG_2	
Tyr-146	5.01 OH–N					6.26 OH–O	6.40 CE_1–C
Leu-149	5.85 CD_1–CB	5.01 CD_1–CG_2					
Tyr-151	5.78 CE_1–O	4.24 CE_2–CG_2					

[a] The first atom notation refers to the neighboring residue and the second atom refers to the site residue (for atom notations, see Watson, 1969). The distances of the nearest-neighbor residues from site 3 residues were calculated from the 2.0 Å coordinates of the SpMb structure (Takano, 1984).

these questions, mAbs are needed whose specificities are predetermined by the investigator and thus are precisely known. This was made possible by the discovery that small peptides (six residues or longer) can be used as immunogens in their free form (i.e., without coupling to any carrier) to generate polyclonal and mAbs of predetermined specificities (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*; Young *et al.*, 1983; for review, see Atassi, 1986). In this study a free synthetic peptide was employed as the immunizing antigen. Since a carrier was not used, no additional unknown residues (from the carrier) would contribute to binding with antibodies against the peptide. Although, clearly, any protein whose covalent and 3D structures are known could be employed as a model for such studies, we have used here SpMb, because we have already made a large number of synthetic Mb peptides and have isolated a number of myoglobins from various species. In addition, these studies will afford the opportunity to check whether it has discontinuous antigenic sites. These had been surmised to exist in Mb entirely from interpretation of cross-reaction results based on the aforementioned assumption (i.e., that replacements causing a change are part of the site).

The nearest neighbor residues of antigenic site 3 (residues 94–100), which represent the immediate (within 7.0 Å) molecular environment of the site are listed in Table II. The replacements in these nearest-neighbor residues in the Mb variants used in this study are given in Table III. The Mb variants (Table I) were selected based on the substitutions within the region 94–100. Some variants had no substitutions in that region while others have substitutions both inside and

Table III. Nearest-neighbor (Environmental) Residues to Antigenic Site 3 of Sperm Whale Mb That Undergo Substitution in Other Myoglobins and the Nature of Substitutions[a]

Antigenic site 3 environment in sperm whale Mb	Environmental residue substitutions in other myoglobins						
	PdMb	FbMb	CsMb	ChMb	DgMb	EdMb	BqMb
Lys-42				Arg			
Phe-3							
Ala-90							
Ser-92							
His-93							
Ala-94							
Thr-95							
Lys-96							
His-97							
Ile-99							
Pro-100							Val
Tyr-103						Ser	
Leu-104						Phe	
Tyr-146							
Leu-149				Phe		Phe	
Tyr-151	Phe	Phe	Phe	Phe	Phe	Phe	Phe

[a] For the distances and molecular contacts of environmental residues within 7 Å from a site residue, see Table II. Distances were calculated from the 2.0 Å coordinates of the SpMb structure (Takano, 1984).

outside the region. The Mb variants will, therefore, be divided according to these substitutions and will be discussed below.

The finding here, that PdMb, FbMb and CsMb, which have no substitutions within the region 94–100 (Table I), exhibited much lower reactivities and relative binding affinities to antisite 3 mAbs than the reference antigen, SpMb, clearly demonstrate that amino acid substitutions outside the region 94–100 can extend their influences to alter the reactivity of that region. If the region 94–100, to which the specificity of these mAbs is directed is not affected by changes elsewhere in the molecule then the cross-reactivities of each of the three myoglobins should be equal to SpMb. The amino acid replacements which are located outside antigenic site 3 and must be responsible for these changes are listed in Table IV, together with their minimum distances from the site (which ranged from 4.24–34.86 Å). Another pair of Mb variants (ChMb and DgMb), which also have no substitutions in site 3 (Table I), exhibited drastic losses in their cross-reactivities with antisite 3 mAbs (Figs. 3 and 5). These dramatic immunochemical changes obviously stemmed from effects exerted by amino acid substitutions outside the region 94–100. These substitutions are listed in Table V, together with their minimum distances from the site which varied from 4.22–33.76 Å.

Of the echidna and badger Mbs, each has a single substitution in site 3 (Table I), in addition to the substitutions elsewhere in the molecule. In this case, it will be uncertain whether to attribute the complete destruction in their cross-reactivities to the single amino acid substitution within the site or to those outside the antigenic site. It is quite likely the result of both. The minimum distances of these substitutions (which varied from 4.24–33.76 Å, from site 3) are given in Table VI.

The observed changes in the immunochemical reactivity of a given Mb variant, relative to SpMb, were most likely due to the cumulative indirect effects of amino acid substitutions outside site 3. The individual contribution of each substitution to the overall effect is difficult to determine but would be a function of the nature of the replacement and its distance from the antigenic site. Further, it should be expected that the extent of influences in a given Mb of amino acid substitutions outside the site is also dependent on the number of amino acid substitutions. This was clear in the binding properties of PdMb, FbMb, and CsMb compared to ChMb and DgMb. The latter suffered more substitutions and were accordingly much more affected. As expected, the patterns of cross-reactivities varied from one monoclonal antibody to another, probably due to some differences in the complementarity residues making up the antibody-combining sites.

Table IV. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Pacific Common Dolphin Mb, Finback Whale Mb, and California Sea Lion Mb and their closest distances (Å) from Site 3 (Residues 94–100) in Sperm Whale Mb.

A Residue no.	Residues in SpMb	Replaced by			Dist. of A to (closest residue of) site 3 in SpMb[a]
		In PdMb	in FbMb	In CsMb	
1	V	G	—	G	26.00 (Ala-94)
3	S	—	T	—	30.72 (Ala-94)
4	E	D	D	D	30.02 (Ala-94)
5	G	—	A	—	30.95 (Ile-99)
8	Q	—	H	—	34.86 (His-97)
12	H	N	N	N	32.17 (Ile-99)
13	V	—	I	I	26.49 (Ile-99)
15	A	G	—	G	27.29 (His-97)
21	V	L	—	L	20.42 (His-97)
22	A	—	—	V	20.17 (His-97)
27	D	—	—	E	21.96 (Ile-99)
28	I	V	—	V	16.55 (Ile-99)
31	R	—	S	—	19.16 (Pro-100)
35	S	G	G	G	16.48 (Pro-100)
45	R	K	K	K	10.91 (His-97)
51	T	—	—	S	22.84 (Ile-99)
53	A	—	—	D	24.72 (Ile-99)
54	E	D	—	—	20.72 (Ile-99)
57	A	—	—	R	23.32 (Ile-99)
66	V	N	N	K	16.85 (His-97)
74	A	—	G	G	16.03 (His-97)
83	E	D	—	—	18.08 (Ala-94)
109	E	—	D	—	13.25 (Ile-99)
116	H	—	—	Q	27.93 (Ile-99)
118	R	—	—	K	24.88 (Ile-99)
121	G	A	A	—	33.76 (Ile-99)
122	D	E	—	—	32.39 (Ile-99)
127	A	—	—	T	27.92 (Ile-99)
128	Q	—	—	H	24.13 (Pro-100)
129	G	—	A	A	24.07 (Pro-100)
132	N	—	—	K	19.97 (Pro-100)
140	K	—	—	N	17.58 (Ala-94)
147	K	—	—	R	14.67 (Ala-94)
151	Y	F	F	F	**4.24 (Thr-95)**
152	Q	H	—	—	11.49 (Ala-94)

[a] These values represent the shortest distance between the residues in column A to the site 94–100. Values in bold type indicate distance less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of the SpMb structure of Takano (1984).

The studies reported here and in the following papers will afford the opportunity to check whether Mb has discontinuous antigenic sites. These had been surmised to exist in Mb (East *et al.*, 1980, 1982; Berzofsky *et al.*, 1982; Benjamin *et al.*, 1984; Berzofsky and Berkower, 1989) entirely from interpretation of cross-reaction results based on the assumption that every replacement that causes a change in immunochemical reactivity is within the boundaries of the antigenic site. Amino acid residues proposed to constitute discontinuous antigenic sites and which are relevant to antigenic site 3 will be discussed here. Those residues that are relevant to the other sites are discussed in the following papers. Amino acid residues Leu-9, Val-13, Ala-53, His-116, Asp-122, and Gly-124 were proposed to be part of a discontinuous antigenic site (East *et al.*, 1980). The present work shows that the effect of these substitutions is exerted indirectly on the binding of site 3 (as well as other sites, see the following papers). From cross-reactions with two mAbs, Ala-53, His-113 and Ala-74, Ile-142 were interpreted to be in discontinuous antigenic sites (East *et al.*, 1982). The present work has revealed that these substitutions exert their effects rather indirectly on site

Table V. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Dog Mb and Chicken Mb and Their Closest Distances (Å) from Site 3 (Residues 94–100) in Sperm Whale Mb

A Residue no.	Residues in SpMb	Replaced by		Dist. of A to (Closest residue of) site 3 in SpMb[a]
		In DgMb	In ChMb	
1	V	G	G	26.0 (Ala-94)
4	E	D	D	30.02 (Ala-94)
5	G	—	Q	30.95 (Ile-99)
9	L	I	Q	30.23 (Ile-99)
12	H	N	T	32.55 (His-97)
13	V	I	I	26.49 (Ile-99)
15	A	G	G	27.29 (His-97)
19	A	T	—	27.31 (His-97)
21	V	L	I	20.43 (His-97)
26	Q	—	H	19.07 (Ile-99)
27	D	E	E	21.96 (Ile-99)
28	I	V	V	16.55 (Ile-99)
30	I	—	M	18.59 (Ile-99)
34	K	—	H	21.08 (Ile-99)
35	S	N	D	16.48 (Pro-100)
41	E	D	D	13.60 (Pro-100)
42	**K**	**—**	**R**	**4.43 (Lys-98)**
45	R	K	K	10.91 (His-97)
48	H	—	G	18.13 (His-97)
53	A	D	P	26.87 (His-97)
54	E	—	D	20.72 (Ile-99)
57	A	G	G	23.32 (Ile-99)
66	V	N	Q	16.85 (His-97)
74	A	G	—	16.03 (His-97)
75	I	—	Q	13.30 (His-97)
85	E	—	D	16.60 (Ala-94)
92	**S**	**—**	**T**	**4.22 (His-97)**
101	I	V	V	8.68 (Pro-100)
109	E	D	-	16.22 (Pro-100)
110	A	—	V	14.19 (Ile-99)
113	H	Q	K	22.04 (Ile-99)
116	H	Q	A	27.93 (Ile-99)
117	S	—	E	24.28 (Ile-99)
118	R	K	K	24.88 (Ile-99)
120	P	S	A	29.87 (Ile-99)
121	G	—	A	33.76 (Ile-99)
124	G	H	-	28.74 (Ile-99)
127	A	T	S	27.92 (Ile-99)
128	Q	E	—	24.13 (Pro-100)
129	G	A	A	24.07 (Pro-100)
132	N	L	K	19.97 (Pro-100)
140	K	N	D	20.50 (Thr-95)
142	I	—	M	8.98 (Ala-94)
144	A	—	S	11.39 (Ala-94)
149	**L**	**—**	**F**	**5.01 (Thr-95)**
151	**Y**	**F**	**F**	**4.24 (Thr-95)**

[a] These values represent the shortest distance between the residues in column A to the site 94–100. Values in bold type indicate distance is less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of SpMb structure of Takano (1984).

Table VI. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Badger Mb and Echidna Mb and Their Closest Distances (Å) from Site 3 (Residues 94–100) In Sperm Whale Mb

A Residue no.	Residues in SpMb	Replaced by		Dist. of A to (Closest residue of) site 3 in SpMb[a]
		In BgMb	In EdMb	
1	V	G	G	26.0 (Ala-94)
4	E	D	D	30.02 (Ala-94)
12	H	N	K	32.17 (Ile-99)
15	A	G	G	27.29 (His-97)
19	A	—	T	27.31 (His-97)
21	V	L	I	20.43 (His-97)
22	A	—	T	20.17 (His-97)
27	D	E	—	21.96 (Ile-99)
28	I	V	V	16.55 (Ile-99)
35	S	G	T	16.48 (Pro-100)
45	R	K	K	10.91 (His-97)
51	T	S	—	22.84 (Ile-99)
53	A	D	—	24.72 (Ile-99)
57	A	G	—	23.32 (Ile-99)
59	E	—	A	21.65 (His-97)
66	V	N	G	16.85 (His-97)
67	T	—	V	9.06 (His-97)
74	A	G	S	16.03 (His-97)
81	H	—	Q	24.07 (Ala-94)
82	H	Q	—	15.63 (Ala-94)
100	P	—	S	5.04 (Ile-99)
101	I	V	—	8.68 (Pro-100)
103	Y	—	F	4.92 (Pro-100)
109	E	D	—	16.22 (Pro-100)
112	I	A	—	19.06 (Pro-100)
113	H	Q	—	22.04 (Ile-99)
116	H	Q	Q	27.93 (Ile-99)
118	R	K	K	24.88 (Ile-99)
120	P	—	S	29.87 (Ile-99)
121	G	—	A	33.76 (Ile-99)
122	D	N	—	32.39 (Ile-99)
124	G	A	—	28.82 (Ile-99)
126	D	E	—	31.41 (Ile-99)
129	G	—	A	24.07 (Pro-100)
132	N	K	G	19.97 (Pro-100)
140	K	N	N	17.58 (Ala-94)
142	I	—	I	8.98 (Ala-94)
149	L	—	F	**5.01 (Thr-95)**
151	Y	F	F	**4.24 (Thr-95)**

[a] These values represent the shortest distance between the residue in column A to the site 94–100. Values in bold type indicate distance is less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of SpMb structure of Takano (1984).

3 (as well as on other sites as seen in the following papers). Similarly, Glu-4, Glu-83, Ala-144, and Lys-140, which had been proposed to reside within discontinuous sites (Berzofsky *et al.*, 1982), their effects were found here to be in nature indirectly exerted on the reactivity of site 3 (as well as other sites as shown in the following papers). The mAbs employed in those studies (East *et al.*, 1982; Berzofsky *et al.*, 1982) were generated by using whole Mb as the immunogen and, therefore, their site specificity was not known but was totally surmised from cross-reaction studies with Mb variants, each of which had many substitutions, such as the Mbs used in the present work. The results reported here and in the following papers with peptide-elicited mAbs clearly show that such interpretations are not correct.

In conclusion, the results with antisite 3 mAbs have shown that amino acid substitutions outside this

region, which by design were not part of the recognition site of antisite 3 mAbs can exert destructive effects, on the binding of that region. Clearly, therefore, it is incorrect to assume that every mutation affecting the binding of a protein to mAbs should be part of an antigenic site.

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β-Endorphin omission analogs: Dissociation of immunor activity from other biological activities

(peptide synthesis/radioimmunoassay/analgesia/ileal opiate activity/opiate receptor-binding activity)

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ABSTRACT An analog of human β-endorphin with omission of four residues at positions 11, 14, 20, and 22 has been synthesized. This analog and other synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 have been assayed for analgesic potency, ileal opiate activity, opiate receptor-binding activity, and immunoreactivity. Results show that deletion of a single amino acid of the β-endorphin molecule outside of the enkephalin segment to give des-Gln^{11}-, des-Thr^{12}-, des-Pro^{13}-, des-Leu^{14}-, des-Val^{15}-, des-Asn^{20}-, or des-Ile^{22}-β-endorphin markedly reduced or abolished the immunoreactivity yet gave substantial retention of opiate potencies. Deletion of a single amino acid of β-endorphin within the enkephalin segment (des-Gly^{5}- or des-Met^{5}-β-endorphin) did not markedly affect the immunoactivity; however, the opiate activities were abolished or markedly reduced. The data indicate a clear dissociation of immunoactivity from analgesic, ileal-opiate, and opiate receptor-binding activities.

β-Endorphin (β-EP) (ref. 1; see Fig. 1) is a naturally occurring opioid peptide with potent opiate analgesic activity after intracerebral (2) or intravenous injections (3, 4). Studies on structure–activity relationships indicate that the entire β-EP molecule is necessary for full analgesic potency (5). In addition, omission of a single amino acid residue at position 14 or 20 abolishes immunoreactivity yet gives retention of opiate potency (6). We present herein biological activities of synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 as well as a synthetic analog with omission of four residues at positions 11, 14, 20, and 22. The analogs were assayed for analgesic activity by the tail-flick test, ileal opiate activity by the guinea pig ileum method, opiate receptor-binding activity by displacement of [^{3}H]-β-EP binding to membrane of rat brain, and immunoreactivity by radioimmunoassay. Results show a clear dissociation of immunoreactivity from other biological activities.

MATERIALS AND METHODS

Synthesis of single-deletion analogs of β_c-EP has been described (6, 7). Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP was synthesized by the solid-phase method (8). It was performed with Boc(Bzl)Glu brominated polymer (0.34 mmol/g) (4) on a Beckman model 990 peptide synthesizer. A fully automated symmetrical anhydride program (5) was used except for the Asn residue, which was incorporated by procedures described for the synthesis of β_h-EP (4). The following amino acid residues in the β_h-EP sequence were omitted in the synthesis: Gln^{11}, Leu^{14}, Asn^{20}, and Ile^{22}. From 295 mg (100 μmol) starting resin there was obtained, after removal of the last Boc group, 768 mg of protected peptide resin corresponding to des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP. Cleavage and deprotection in HF (9), gel filtration on Sephadex G-10 (0.5 M acetic acid), and chromatography on carboxymethylcellulose were performed as described (4). From additivity rules for the hydrophobicities of amino acid residues in β-EP (7) it could be predicted that partition chromatography on Sephadex G-50 in the same solvent system used for β_h-EP (4) would give an R_F of 0.27 based on the reported R_F of 0.40 for β_h-EP. The experimental value of R_F was found to be 0.26. The overall yield of des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP based on 50 μmol of starting resin was 39.0 mg (26%). The product (50-μg samples) was homogeneous on thin-layer chromatography on silica gel in 1-butanol/pyridine/acetic acid/H_2O (5:5:1:4, vol/vol), R_F 0.40 (ninhydrin and chlorine–tolidine detection), and in paper electrophoresis on Whatman 3 MM at pH 3.7 (R_F 0.56 relative to Lys) and pH 6.7 (R_F 0.45 relative to Lys) at 400 V (5 hr, ninhydrin detection). Amino acid analysis of a 24-hr HCl hydrolysate gave (theoretical values in parentheses): Lys, 4.91 (5); Asp, 1.06 (1); Thr, 3.12 (3); Ser, 1.92 (2); Glu, 2.10 (2); Pro, 0.96 (1); Gly, 2.97 (3); Ala, 2.12 (2); Val, 1.02 (1); Met, 0.98 (1); Ile, 1.00 (1); Leu, 1.07 (1); Tyr, 1.96 (2); Phe, 1.96 (2). Amino acid analysis of an enzymic digest (trypsin and chymotrypsin followed by leucine aminopeptidase) gave: Lys, 4.90 (5); Thr + Ser + Asn, 6.30 (6); Glu, 1.96 (2); Pro, 0.91 (1); Gly, 2.84 (3); Ala, 2.14 (2); Val, 1.12 (1); Met, 0.98 (1); Ile, 1.06 (1); Leu, 1.10 (1); Tyr, 1.90 (2); Phe, 1.84 (2).

```
                    5                   10
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-

                   15                  20
Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                   25                      31
Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
```

FIG. 1. Amino acid sequence of β_h-EP (β_c-EP: His-27, Gln-31). Residues 1–5 correspond to [Met]enkephalin.

Opiate activities were assessed both *in vitro* and *in vivo*. The ileal opiate activity *in vitro* was measured by the inhibition of electrically stimulated contraction of guinea pig ileum preparation (10), and the opiate receptor-binding assay was performed according to the procedure recently described (11, 12), using [^{3}H-Tyr^{27}]-β_h-EP (13) as the primary ligand and synthetic

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Abbreviations: β-EP, β-endorphin (subscripts h and c indicate β-EP from human and camel pituitaries); IC_{50}, 50% inhibitory concentration.

Table 1. Ileal opiate activities of omission analogs of β-EP

Synthetic peptide	IC_{50},* nM	Relative potency
β_c-EP	91	100
Des-Gly^2-β_c-EP	9100	1
Des-Leu^{14}-β_c-EP	65	140
β_c-EP	58	100
Des-Thr^6-β_c-EP	60	97
Des-Ser^{10}-β_c-EP	49	118
Des-Thr^{12}-β_c-EP	50	116
β_c-EP	132	100
Des-Met^5-β_c-EP	2000	7
Des-Val^{15}-β_c-EP	107	123
Des-Ile^{22}-β_c-EP	67	197
β_h-EP	22	100
β_c-EP	22	100
Des-Gln^{11}-β_c-EP	22	100
Des-Pro^{13}-β_c-EP	20	110
Des-Asn^{20}-β_c-EP	20	110
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	16	138

* Guinea pig ileum assay; IC_{50} is the concentration that gives 50% inhibition of contraction.

β_c-EP (14) or β_h-EP (4) as standard competing ligand. The analgesic activity *in vivo* was assessed in mice by the tail-flick method (15) as described (5). Radioimmunoassay was carried out by the procedure described (16, 17).

RESULTS

The opiate activities *in vitro* of various omission analogs as assayed by the guinea pig ileum preparation are summarized in Table 1. Deletion of Gly^2 or Met^5 in the [Met]enkephalin segment of β-EP causes a marked decrease of opiate potency. On the other hand, deletion of a single amino acid residue outside the [Met]enkephalin segment does not alter the opiate potency and in some cases even enhances it. For example, des-Ile^{22}- and des-Leu^{14}-β_c-EP are 1.4 and 1.97 times as potent as the intact peptide, respectively. Omission of four residues in positions 11, 14, 20, and 22 increases the potency to 138% compared with β_h-EP.

Table 2. Analgesic potencies of omission analogs of β-EP

Synthetic peptide	AD_{50}*, nmol/mouse	Relative potency
β_c-EP	0.026 (0.020–0.032)	100
Des-Gly^2-β_c-EP	>25	<0.1
Des-Gln^{11}-β_c-EP	0.033 (0.021–0.048)	79
Des-Pro^{13}-β_c-EP	0.113 (0.089–0.149)	23
Des-Asn^{20}-β_c-EP	0.057 (0.042–0.075)	46
β_c-EP	0.043 (0.035–0.075)	100
Des-Met^5-β_c-EP	0.219 (0.099–0.487)	20
Des-Thr^6-β_c-EP	0.059 (0.048–0.077)	73
Des-Ser^{10}-β_c-EP	0.047 (0.027–0.092)	92
Des-Thr^{12}-β_c-EP	0.045 (0.024–0.095)	96
Des-Leu^{14}-β_c-EP	0.057 (0.033–0.093)	75
Des-Val^{15}-β_c-EP	0.179 (0.131–0.241)	24
Des-Ile^{22}-β_c-EP	0.075 (0.045–0.117)	57
β_h-EP	0.064 (0.026–0.17)	100
Des-Gln^{11},Leu^{14},Asn^{20}, Ile^{22}-β_h-EP	0.99 (0.45–2.19)	7

* Median antinociceptive dose (95% confidence limit).

Table 3. Opiate receptor-binding activities of omission analogs of β-EP

Synthetic peptide	IC_{50}, pM	Relative potency
β_c-EP	250	100
Des-Gly^2-β_c-EP	50,000	0.5
Des-Met^5-β_c-EP	12,000	2
Des-Thr^6-β_c-EP	550	45
Des-Ser^{10}-β_c-EP	280	90
Des-Gln^{11}-β_c-EP	210	120
Des-Thr^{12}-β_c-EP	270	93
Des-Pro^{13}-β_c-EP	860	29
Des-Leu^{14}-β_c-EP	430	58
Des-Val^{15}-β_c-EP	390	64
Des-Asn^{20}-β_c-EP	530	47
Des-Ile^{22}-β_c-EP	180	139
β_h-EP	560	100
β_c-EP	250	224*
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	610	92*, 41†

* Relative to β_h-EP.
† Relative to β_c-EP.

Table 2 presents the analgesic potencies *in vivo* of the synthetic analogs. Des-Gly^2-β_c-EP is less than 0.1% as potent as β_c-EP, and des-Met^5-β_c-EP exhibits only 20% potency relative to that of β_c-EP. The majority of analogs with deletion of a single amino acid residue outside the [Met]enkephalin segment retain substantial analgesic potency. However, deletion of four residues (positions 11, 14, 20, and 22) drops potency to 7% of that of the intact molecule.

As in the ileal opiate activity assay, the deletion of a single amino acid residue at position 2 or 5 markedly reduced the potency in the opiate receptor assay using membranes of rat brain. Deletion of a single amino acid residue outside the enkephalin segment, however, did not markedly alter the opiat receptor-binding potency (Table 3). Even deletion of four residues simultaneously had no drastic effects.

Table 4 summarizes the immunoreactivity of omission analogs by the β_h-EP radioimmunoassay system. Deletion of a single amino acid residue at position 11, 12, 13, 14, 15, or 20 abolished or markedly reduced the abilities of these peptides

Table 4. Immunoactivity of omission analogs of β-EP

Synthetic peptide	IC_{50},* pM	Relative activity
β_c-EP	72	100
Des-Gln^{11}-β_c-EP	800	9
Des-Pro^{13}-β_c-EP	1,029	7
Des-Leu^{14}-β_c-EP	>10,000	<1
Des-Val^{15}-β_c-EP	>10,000	<1
Des-Asn^{20}-β_c-EP	>10,000	<1
Des-Ile^{22}-β_c-EP	248	29
β_c-EP	56	100
Des-Gly^2-β_c-EP	68	82
β_h-EP	51	100
β_c-EP	51	100
Des-Met^6-β_c-EP	67	76
Des-Thr^6-β_c-EP	48	106
Des-Ser^{10}-β_c-EP	70	73
Des-Thr^{12}-β_c-EP	1,300	3
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	>10,000	<1

* Radioimmunoassay.

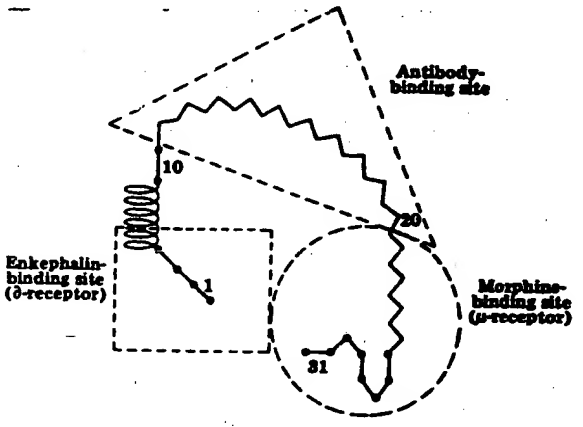
t bind to the antibodies of β-EP. On the other hand, omission of Gly^2 or Met^5 in the [Met]enkephalin segment of β_c-EP as well as Thr^6 or Ser^{10} resulted in retention of high immunoreactive potency.

DISCUSSION

Previous studies indicated the importance of the Tyr^1, Phe^4, and Met^5 residues for the production of opiate analgesic activity (18–20). In this study, we found that deletion of Gly^2 or Met^5 in the [Met]enkephalin segment of β-EP drastically lowers opiate analgesic, ileal opiate, and receptor-binding potency, whereas nearly full immunoreactivity is retained. Of these two residues, Gly^2 appears to be more important for the production of opiate activities. Omission of a single residue outside of this segment does not cause considerable loss of biological activities, but immunoreactivity is markedly affected. Omission of a single amino acid at position 14, 15, or 20 abolishes immunoreactivity yet retains significant amounts of other biological activities. This indicates that the active sites in the β-EP molecule for binding to the β-EP antibodies resides in positions 11 to 22. Thus we have discovered an instance in which deletion of a single amino acid residue in a biologically active peptide abolishes immunoreactivity.

Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP has virtually no immunoreactivity and exhibits somewhat higher ileal opiate and significant receptor-binding activity in comparison to β_h-EP. Analgesic potency of this analog is only 7% when compared with the activity for the intact molecule. In an earlier report (16), a lack of correlation between immunoreactivity and opiate activity as assayed by the guinea pig ileum preparation has been noted.

When the relative ileal opiate activities for des-Gln^{11}-, des-Leu^{14}-, des-Asn^{20}-, and des-Ile^{22}-β_c-EP are averaged, a value of 137 is obtained. It is interesting that the observed opiate activity of des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP is 138. On the other hand, the similarly calculated values for the other activities of des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP (receptor-binding activity, 124; analgesic potency, 64, and immunoreactivity, 10) diverge increasingly from the experimental data in the order given. These results illustrate the insensitivity of the ileal assay to such structural alterations, while the other assays show sensitivity in the order immunoreactivity > analgesic activity > receptor-binding activity.



, Random coil; , α helix; , β sheet; , β turn

FIG. 2. Proposed binding sites in the primary structure of β-EP. Predicted secondary structure of β_h-EP was taken from ref. 23.

The data, summarized in Table 5, clearly show the dissociation of immunoreactivity from analgesic, ileal opiate, and receptor-binding activities. Moreover, there is a fair correlation between analgesic potency and receptor-binding activity if des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP is excluded. This omission analog possesses significant receptor-binding activity and low analgesic potency. The lack of correlation between opiate receptor-binding activity and analgesic potency has recently been observed with synthetic analogs with extension at the COOH terminus (21). These data emphasize again the importance of not relying on a single assay procedure for the characterization of biologically active peptides.

There are at least two receptors for opioid peptides in the brain (22): the μ receptors for morphine and the ∂ receptors for the enkephalins. The data presented herein, together with the recent findings that β_c-EP-(6–31) and β_c-EP-(20–31) segments inhibit morphine-induced analgesia (unpublished), suggest the presence of three binding sites in the β-EP molecule as shown in Fig. 2. The first site resides in the [Met]enkephalin segment [enkephalin-binding site ("∂-receptor")] and the second consists

Table 5. Relative biological activities of omission analogs of β-EP

Synthetic peptide	Analgesic potency	Opiate activity	Receptor-binding activity	Immunoreactivity
β_c-EP	100	100	100	100
Des-Gly^2-β_c-EP	<0.01	1	0.5	82
Des-Met^5-β_c-EP	20	7	2	76
Des-Thr^6-β_c-EP	73	97	45	106
Des-Ser^{10}-β_c-EP	92	118	90	73
Des-Gln^{11}-β_c-EP	79	100	120	9
Des-Thr^{12}-β_c-EP	96	116	93	3
Des-Pro^{13}-β_c-EP	23	110	29	7
Des-Leu^{14}-β_c-EP	75	140	58	<1
Des-Val^{15}-β_c-EP	24	123	64	<1
Des-Asn^{20}-β_c-EP	46	110	47	<1
Des-Ile^{22}-β_c-EP	57	197	139	29
β_h-EP	100	100	100	100
β_c-EP	100	100	224*	100
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	7	138	92*, 41†	<1

* Relative to β_h-EP.
† Relative to β_c-EP.

of the COOH-terminal segment [β-EP-(21–31)] [m rphine-binding sit ("μ-receptor")]. The middle segment [β-EP-(11–20)] is the antibody-binding site. Studies on the *in vivo* and *in vitro* biological profiles of synthetic β-EP analogs may possibly clarify the role of these binding sites.

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Anti-PDI

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Rabbit Anti-Protein Disulfide Isomerase Polyclonal Antibody
Product #: SPA-890

Immunogen
Purified bovine liver Protein Disulfide Isomerase (PDI).

Specificity
This antibody identifies purified bovine PDI protein and will detect an ~58 kDa protein, corresponding to the apparent molecular mass of PDI on SDS-PAGE immunoblots, in samples from human, mouse, rat, monkey, hamster, guinea pig, bovine, sheep, pig, dog, and *Xenopus* origins. This antibody identifies purified bovine PDI protein.

Species Reactivity
human, mouse, rat, monkey, hamster, guinea pig, bovine, sheep, pig, dog, *Xenopus*

Applications	Certified*	Cited
Western Blot (Colorimetric) (4,5,8,9,11)	1:4,000	
Western Blot (ECL) (12,13)		1:5,000
Immunoprecipitation (11,16)		1:100
Immunocytochemistry (6,10,11,14,15)		Yes

*These working dilutions are provided as suggestions only. Further dilutions may be possible. Each user should determine the optimal conditions for their own particular experiment.

Positive Controls
Purified Bovine PDI Protein, Product#: SPP-890
HeLa Cell Lysate, Product#: LYC-HL100

Scientific Background
The mammalian protein disulphide-isomerase (PDI) family encompasses several highly divergent proteins which are involved in the processing and maturation of secretory proteins in the ER by catalyzing the rearrangement of disulphide bonds (2,7). PDI, which is an abundant protein of the ER (>400uM), has a carboxy-terminal retention signal sequence, KDEL, similar to that of BiP and Grp94 (1). The PDI proteins are characterized by the presence of one or more domains of ~95-110 amino acids related to the cytoplasmic protein thioredoxin (2). All but the PDI-D subfamily are composed entirely of repeats of such domains, with at least one domain containing and one domain lacking a redox-active-Cys-Xaa-Xaa-Cys-tetrapeptide (2). In addition to their roles as redox catalysts and isomerases, PDI proteins have other functions such as peptide binding, cell adhesion and perhaps chaperone activities (2). Platelet surface thiols and disulphides play an important role in platelet responses (3). Catalytically active PDI is found on platelet surfaces where it has been demonstrated to mediate platelet aggregation and secretion possible by reducing disulfide bonds thus leading to exposure of fibrinogen receptors in platelets (3).

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Rev: 03/11/03

CERTIFICATE OF ANALYSIS

Anti-PDI

Product #: SPA-890 **Size:**

Lot #: B302407
Format: Whole rabbit serum

Host: Rabbit **Antibody Type:** Polyclonal
Certification: A 1:4,000 dilution of SPA-890 was sufficient for detection of PDI in 50ng of purified bovine PDI protein (product# SPP-890) by colorimetric immunoblot analysis using Stressgen's Goat anti-Rabbit IgG:AP (product# SAB-301) as the secondary antibody.

Certified by: K. Levant **QC by:** C. Franz
Date: 03/11/03 **Date:** 02/14/03

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This product is sold only for research use by qualified laboratory personnel, and is not to be used as a drug, medical device, food additive, cosmetic, nor household chemical. It is not to be used in diagnostic, therapeutic, consumer, agricultural, nor pesticidal applications.

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Section 5 - Reactivity Data

NOT APPLICABLE

Section 6 - Toxicological Properties

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Wear chemical safety goggles and compatible chemical-resistant gloves. Avoid inhalation, contact with eyes, skin or clothing.

*****MULTIPLE COMPONENT SPILL OR LEAK PROCEDURES*****

- Wear protective equipment.
- Absorb on sand or vermiculite and place in closed containers for disposal.
- Dispose or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber. Observe all federal, state and local environmental regulations.

Section 8 - First Aid Measures

- If swallowed, wash out mouth with water, provided person is conscious. Call a physician.
- In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes. If a rash or other irritation develops, call a physician.
- If inhaled, remove to fresh air. If breathing becomes difficult, call a physician.
- In case of eye contact, flush with copious amounts of water for at least 15 minutes while separating the eyelids with fingers. Call a physician.

Section 9 - Preparation

Prepared by:	Phone#:	Created:	Modified:
SBC	(250) 744-2811	03/11/03	03/11/03

PDI Antibody - Rabbit Polyclonal

Catalog Number	Format	Tested Applications	Size	Price	Quantity to Add to Cart
SPA-890D	Antiserum	WB, IP, ICC	50ul	$144.00	1 Add
SPA-890F	Antiserum	WB, IP, ICC	200ul	$319.00	1 Add

This antibody detects an ~58 kDa band corresponding to the apparent molecular mass of Protein Disulfide Isomerase (PDI) on SDS-PAGE immunoblots.

Tech Spec Download

Immunogen Bovine PDI

Antigen The predicted molecular weight of the antigen, 57,265 (precursor protein) Daltons, is calculated from the sequence reported in M17596 in the GenBank database (unless stated otherwise). This information is provided as a guide only; empirical results may differ.

Reactivity human, mouse, rat, bovine, canine, guinea pig, hamster, monkey, pig, sheep, Xenopus

Positive Controls HeLa Cell Lysate, LYC-HL100

Recommended Secondary Antibodies Anti-Rabbit Total IgG, Conjugated to HRP, AP, Biotin, or FITC

See our selection of secondary antibodies.

Search for Citations **of this product**

List the Product Groups **this product belongs to.**

Add Additional Products

1: M17596. Bovine protein di...[gi:163496]

Links

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DEFINITION  Bovine protein disulfide isomerase (PDI) mRNA, complete cds.
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VERSION     M17596.1  GI:163496
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  AUTHORS   Yamauchi,K., Yamamoto,T., Hayashi,H., Koya,S., Takikawa,H.,
            Toyoshima,K. and Horiuchi,R.
  TITLE     Sequence of membrane-associated thyroid hormone binding protein
            from bovine liver: its identity with protein disulphide isomerase
  JOURNAL   Biochem. Biophys. Res. Commun. 146 (3), 1485-1492 (1987)
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   PUBMED   3619939
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  AUTHORS   Yamauchi,K., Yamamoto,T., Hayashi,H., Koya,S., Takikawa,H.,
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  TITLE     Sequence of membrane-associated thyroid hormone binding protein
            from bovine liver: its identity with protein disulphide isomerase
  JOURNAL   Biochem. Biophys. Res. Commun. 146 (3), 1485-1492 (1987)
  MEDLINE   87298601
   PUBMED   3619939
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 SeqServer Version 4.6 Jan 2002

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- [ ] GI:163497

CLUSTAL W (1.7) Multiple Sequence Alignments

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Sequences (1:2) Aligned. Score:  11
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Aligning...
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Submit sequences to: BLAST2 ▼ Submit Reset

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

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Reticulocalbin, a Novel Endoplasmic Reticulum Resident Ca^{2+}-binding Protein with Multiple EF-hand Motifs and a Carboxyl-terminal HDEL Sequence*

(Received for publication, June 23, 1992)

Masayuki Ozawa‡ and Takashi Muramatsu

From the Department of Biochemistry, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890, Japan

A novel Ca^{2+}-binding protein, tentatively designated reticulocalbin, has been identified and characterized. R ticulocalbin is a luminal protein of the endoplasmic reticulum with an M_r of 44,000 as revealed by biochemical analysis and immunofluorescence staining. The cDNA of reticulocalbin encodes a protein of 325 amino acids with an amino-terminal signal sequence of 20 amino acids. The protein has six repeats of a domain containing the high affinity Ca^{2+}-binding motif, the EF-hand. Although oxygen-containing amino acids important for the positioning of Ca^{2+} are conserved in all six domains, the conserved glycine residues in the central portion of the EF-hand motif are absent in three of them. Calcium blots showed that recombinant reticulocalbin expressed in bacterial cells binds Ca^{2+}. The protein has the sequence His-Asp-Glu-Leu (HDEL) at its carboxyl terminus. This is similar to the Lys-Asp-Glu-Leu sequence, which serves as a signal to retain the resident proteins in the endoplasmic reticulum of animal cells. A mutant protein lacking the HDEL sequence produced by *in vitro* mutagenesis has been shown to be secreted into medium in transient expressi n assays.

Ca^{2+} is involved in the regulation of various cellular activities such as contraction, secretion, and mitogenesis. These events are largely mediated by a family of homologous Ca^{2+}-binding proteins including calmodulin, troponin C, calbindins, and S-100 proteins. These proteins exhibit a common structural motif, the EF-hand (Kretsinger, 1980), which is present in multiple copies (calmodulin and troponin C have four and S-100 proteins have two copies per molecule) and binds Ca^{2+} selectively, with high affinity. Each of these consists of a loop of 12 amino acids that is flanked by two α-helices (Strynadka and James, 1989; Heizman and Hunziker, 1991). Upon Ca^{2+} binding, these proteins undergo a conformational change and consequently interact with their target proteins.

The lumen of the endoplasmic reticulum (ER)[1] (reticuloplasm) contains a number of soluble proteins including immunoglobulin heavy chain-binding protein (GRP78) (Munro and Pelham, 1986; Bole *et al.*, 1986), protein disulfide-isomerase (Freedman, 1989; Edman *et al.*, 1985), and GRP94 (endoplasmin, ERp99) (Mazzarella and Green, 1987; Sorger and Pelham, 1987). Many of these proteins are involved in the initial steps of the maturation of newly synthesized secretory proteins such as folding of nascent polypeptide chains and formation of the correct disulfide bonds. Retention of these resident proteins in the ER is dependent on a carboxyl-terminal signal, which in animal cells is usually Lys-Asp-Glu-Leu (KDEL). The KDEL sequence is recognized by a membrane-bound receptor that continually retrieves the proteins from a later compartment (*cis*-Golgi cisternae) of the secretory pathway and returns them to the ER (Pelham, 1989, 1990). Although the above-mentioned proteins (immunoglobulin heavy chain-binding protein, protein disulfide-isomerase, and GRP94) reportedly bind Ca^{2+} (Macer and Koch, 1988), none of them have the EF-hand motifs.

Calreticulin, a well-characterized Ca^{2+}-binding protein of the ER and the sarcoplasmic reticulum, binds Ca^{2+} with high affinity, but does not have an EF-hand motif (Smith and Koch, 1989; Fliegel *et al.*, 1989). Calreticulin may be a nonmuscle functional analogue of calsequestrin, a major Ca^{2+}-binding (storage) protein of the skeletal muscle sarcoplasmic reticulum membrane (Milner *et al.*, 1991). So far, there have been no reports of ER resident proteins having EF-hand motifs.

We previously isolated several independent cDNA clones from λgt11 libraries of mouse teratocarcinoma OTT6050 (Ozawa *et al.*, 1988; Furukawa *et al.*, 1990). These clones were isolated by screening the libraries with antibodies against *Dolichos biflorus* agglutinin-binding glycoproteins. The lectin *D. biflorus* agglutinin is known to bind specifically with the nonreducing terminal *N*-acetylgalactosamine of carbohydrate chains (Etzler, 1972). Although the majority of the clones showed developmentally regulated expression, others did not, suggesting that the latter are products of housekeeping genes. Sequencing a group of cDNA clones that belong to the latter category and characterizing the protein encoded by these clones revealed that the protein is an ER resident Ca^{2+}-binding protein with multiple EF-hand motifs for which we propose the nomenclature reticulocalbin.

MATERIALS AND METHODS

cDNA Cloning—cDNA clones O1, O9, and O32 were isolated from a λgt11 cDNA library of mouse teratocarcinoma OTT6050 (Ozawa *et al.*, 1988) by screening with antibodies against *D. biflorus* agglutinin-binding glycoproteins as previously described (Ozawa *et al.*, 1988). Clones M10 and M22 were obtained from a primer extension cDNA library in λgt10 and subcloned into pUC18 and Bluescript KS(+)

* This work was supported by grants from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D13003.

‡ To whom correspondence should be addressed. Tel.: 81-992-64-2211; Fax: 81-992-64-5618.

[1] The abbreviations used are: ER, endoplasmic reticulum; bp, base pair(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ConA, concanavalin A; PAGE, polyacrylamide gel electrophoresis; MBP, maltose-binding protein.

(Stratagene) vectors, and the DNA was sequenced from both strands as described (Ozawa *et al.*, 1988).

Construction of Expression Vectors—To express reticulocalbin in animal cells, the cDNA was cloned into a mammalian expression vector, pCAGGS, which contains an enhancer derived from cytomegalovirus and the β-actin promotor (Niwa *et al.*, 1991). The Bluescript KS(+) vector containing the 1052-bp 5′-fragment of reticulocalbin cDNA in the *Eco*RI-*Pst*I site was restricted with *Sma*I and *Hinc*II. The fragment was isolated and cloned into pCAGGS, which had been digested with *Eco*RI and filled in with T4 DNA polymerase. The orientation of the cDNA in the vector was confirmed by restriction enzyme digestion. We constructed an expression vector encoding a mutant reticulocalbin lacking the carboxyl-terminal HDEL peptide by polymerase chain reaction (PCR). Oligonucleotides GACATCGACAAGAACGG and CCTGCAGTCAATTTTTGGTCAGGTCTTCC were synthesized and used as primers. The former corresponds to the sequence of the cDNA from positions 656 to 672. The latter contains a complementary sequence of the cDNA at positions 979–997 and the TGA termination codon as well as a *Pst*I recognition sequence at the 5′-end. The template was the 1052-bp reticulocalbin cDNA in the Bluescript vector, which was linearized by digestion with *Pvu*II. PCR was performed according to the manufacturer's instructions using the GeneAmp PCR reagent kit (Perkin-Elmer Cetus Instruments). The reaction mixture was subjected to 25 cycles of denaturation (93 °C, 1 min), annealing (50 °C, 2 min), and extension (72 °C, 3 min). The PCR product was purified on agarose gel, digested with *Pst*I, and cloned into the *Sma*I-*Pst*I site of Bluescript KS(+). After confirming the sequence of the 3′-*Bam*HI-*Pst*I fragment, the 5′-region was replaced with the authentic fragment and cloned into the pCAGGS vector as described above.

To express recombinant reticulocalbin in *Escherichia coli*, the cDNA encoding the mature protein was cloned into the maltose-binding protein fusion vector (pMAL-c) (New England BioLabs, Inc.) as follows. The cDNA region coding for the signal sequence was eliminated by PCR. We synthesized oligonucleotide CAGCTGCGGGCCAAGCCCACG for use as a primer. It corresponds to the nucleotide sequence encoding the first 6 amino acid residues of the mature protein with the 5′-extension of CAG, which, together with CTG, constitutes a recognition sequence for *Pvu*II. The second primer was oligonucleotide CAGGAAACAGCTATGAC, which was purchased commercially as a primer for dideoxy sequencing (the reverse primer; Takara, Kyoto, Japan). The template, the PCR program, and other conditions were the same as described above. The PCR product was cloned into the *Sma*I-*Pst*I site of Bluescript KS(+) after the fill-in reaction and *Pst*I digestion, and the sequence was confirmed. A 959-bp *Pvu*II-*Pst*I fragment that encodes the entire mature protein was cloned into the *Stu*I-*Pst*I site of the pMAL-c vector. The plasmid DNA was introduced into TB1 cells.

Cells—The mouse parietal endodermal cell line PYS-2, L cells, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 10% CO_2 atmosphere at 37 °C. Genes were transfected by the DEAE-dextran method. Ten micrograms of DNA was mixed with DEAE-dextran (400 μg/ml) and added to COS cells (3×10^6) that were plated 1 day in advance. After 4 h, the cells were washed and incubated with 100 μM chloroquine for 3 h. Cells were cultured for 2 days and analyzed.

Biochemical Analysis—Immunoblot analysis was carried out as described before (Ozawa *et al.*, 1989). Calcium blotting was performed as previously described (Maruyama *et al.*, 1984). For immunoprecipitation, 5×10^6 cells were preincubated for 30 min in Dulbecco's modified Eagle's medium without methionine and 10% dialyzed fetal calf serum and subsequently labeled with [^{35}S]methionine (1000 Ci/mmol; Du Pont-New England Nuclear) at 100 μCi/ml for 30 min. Cells were washed with normal Dulbecco's modified Eagle's medium with 10% fetal calf serum and 2 mM cold methionine and incubated in this medium for 2 h. Cells were washed with PBS and lyzed with PBS containing 1% Nonidet P-40, 1 mM $CaCl_2$, and phenylmethylsulfonyl fluoride. After centrifugation, cell lysates or media were incubated with anti-reticulocalbin antibodies, and immunocomplexes were collected by protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) as previously described (Ozawa *et al.*, 1989). Lectin-agarose fractionation proceeded as follows. Cell lysates were applied to columns (0.4-ml bed volume) containing concanavalin A (ConA)-Sepharose (Pharmacia) or *Ricinus communis* agglutinin-agarose (EY Laboratories) equilibrated with PBS containing 0.1% Nonidet P-40, 1 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride. After washing with 10 ml of this buffer, materials bound to the resin were eluted with the same buffer containing 0.2 M α-methylmannoside (for ConA-Sepharose) or 0.1 M lactose (*R. communis* agglutinin-agarose). The bound and unbound materials were analyzed by SDS-PAGE and immunoblot analysis. For subcellular fractionation, cells were homogenized in 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose. The suspension was centrifuged sequentially at 900 × *g* for 10 min, 5000 × *g* for 10 min, and 100,000 × *g* for 60 min. The precipitates from each centrifugation were designated nuclear, mitochondrial, and microsomal fractions, respectively. The supernatant after the third centrifugation was retained as the soluble fraction. The microsome fraction was further solubilized with Triton X-100 (1%) or sonicated, and the supernatants after centrifugation at 140,000 × *g* for 1 h were analyzed. Phase separation of reticulocalbin in Triton X-114 was performed as described (Bordier, 1981).

Purification of Recombinant Reticulocalbin—Bacterial cells containing the fusion plasmid were cultured, and the fusion protein was induced and collected according to the manufacturer's instructions (New England BioLabs, Inc.). The cells were disrupted by sonication in PBS containing 1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride and then centrifuged. The supernatant was applied to a column of amylose resin and washed with 5–10 column volumes of the following buffers: PBS containing 0.1% Tween 20, PBS containing 0.5 M NaCl and 0.1% Tween 20, and PBS. The fusion protein was eluted with PBS containing 10 mM maltose.

Antibodies—Antibodies were raised against *D. biflorus* agglutinin-binding glycoproteins from teratocarcinoma OTT6050 as described (Ozawa *et al.*, 1982). Monospecific antibodies against reticulocalbin were prepared as follows. The recombinant reticulocalbin of the MBP fusion described above was electrophoresed to remove any contaminating bacterial proteins. The corresponding band was excised from the gels after Coomassie Blue staining and used for immunization in rabbits. The antibodies were affinity-purified by coupling MBP or the fusion protein (1 mg each) to 1 ml of CNBr-activated Sepharose. Antibodies bound to the resin were eluted with 0.1 M glycine HCl (pH 2.5) and immediately neutralized with Tris-HCl (pH 8.8). In some experiments, antibodies eluted from MBP were used as a control.

Immunofluorescence—Cells on coverslips were washed with PBS containing 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$ and fixed with 3% formaldehyde in PBS for 15 min at room temperature. After washing with PBS and incubation with 50 mM NH_4Cl in PBS, cells were permeabilized by incubating with 0.1% Triton X-100 in PBS for 5 min. Cells were preincubated with a mixture (1:1) of PBS and Dulbecco's modified Eagle's medium with 10% fetal calf serum for 15 min and incubated sequentially with anti-reticulocalbin antibodies and goat anti-rabbit antibodies conjugated with fluorescein isothiocyanate (Jackson Laboratories) in the same solution for 30 min. For double staining, biotinylated ConA and rhodamine-labeled avidin (EY Laboratories) were included in the solutions containing anti-reticulocalbin antibodies or anti-rabbit antibodies, respectively. Stained cells were photographed with a Nikon microscope using Fujichrome 100 film. Images from the same slides were generated by a confocal scanning laser microscope (MRC500, Bio-Rad) on a video monitor and photographed using TriX film.

RESULTS

Isolation of Reticulocalbin cDNA Clones—Fig. 1 shows the scheme of isolated reticulocalbin cDNA clones. Clones O1, O9, and O32 were isolated from a cDNA library in λgt11 constructed from mouse teratocarcinoma OTT6050 cDNA by screening with antibodies against *D. biflorus* agglutinin-binding glycoproteins of the cells. These clones provide the 3′-sequence to the internal *Eco*RI site at nucleotide 476, but they did not extend further because *Eco*RI sites were not methylated during construction of the library. Clone O32–8 was obtained by screening a λgt11 library constructed with a cDNA, whose internal *Eco*RI sites were protected with *Eco*RI methylase, by plaque hybridization using the *Eco*RI-*Bam*HI fragment of clone O32 as a probe. To isolate clones M10 and M22, we used an oligonucleotide (30-mer) corresponding to nucleotides 625–655 of reticulocalbin cDNA as a primer for construction of another cDNA library. This library in λgt10 was screened by plaque hybridization using the 144-bp *Eco*RI-*Nde*I fragment of clone O32 as a probe. Northern hybridization of RNAs from teratocarcinoma OTT6050, embryonal

carcinoma cell line F9, and parietal endodermal cell line PYS-2 revealed that the cDNA clones hybridized a major mRNA species of 2.3 kilobase pairs and a minor species of 2.0 kilobase pairs (data not shown).

The composite nucleotide sequence of the cDNA clones and the deduced amino acid sequence of reticulocalbin are presented in Fig. 2. The nucleotide sequence contains 34 bp of the 5′-untranslated region and a 3′-untranslated region of 1016 bp. The coding region specifies a protein of 325 residues with an M_r of 38,112. The amino acid sequence of the first 20 amino acids of reticulocalbin has the typical features of a secretory leader peptide. There is a positively charged amino-terminal region and a hydrophobic central section followed by two small nonpolar residues with a single residue between them. This latter feature is thought to determine the cleavage point of the signal peptidase, which in this case could be between Ala-20 and Leu-21. Cleavage of this peptide would yield a mature protein of 305 residues with a predicted M_r of 36,200. The sequence of reticulocalbin does not contain a hydrophobic transmembrane segment, which suggests transfer of the entire protein into the lumen of the ER. There is one consensus site for *N*-linked glycosylation and a number of serine- or threonine-rich regions that could be *O*-glycosylated.

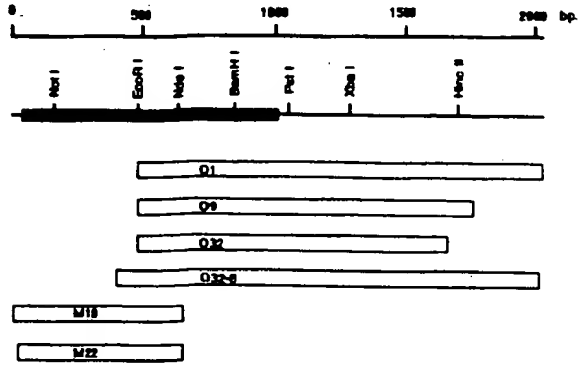
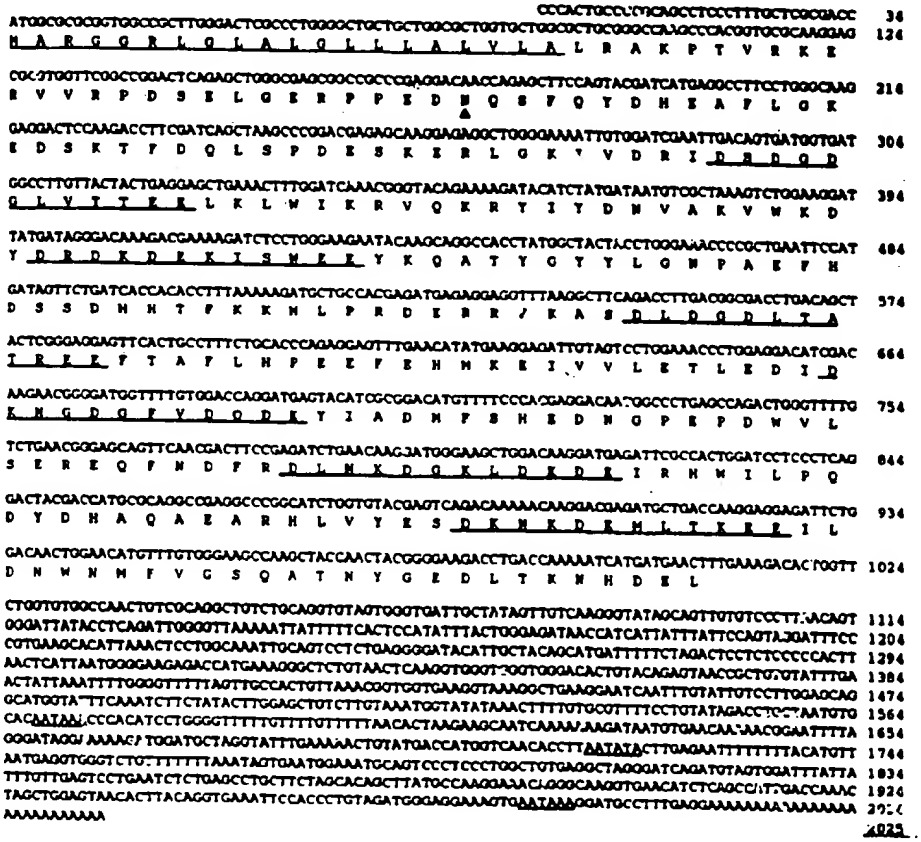


FIG. 1. **Restriction map and clones of reticulocalbin.** The scale indicates nucleotide positions beginning from the first base of clone M10. Beneath the scale, reticulocalbin mRNA is represented by a *thick black line* delineating the coding region. A partial restriction map is indicated. The *open boxes* correspond to the partial cDNA clones isolated from cDNA libraries.

Immunoblots of PYS-2 cells with antibodies against reticulocalbin revealed a band of 44 kDa and a faint band of 46 kDa (Fig. 3). The same bands were also detected in mouse fibroblast L cells (data not shown). The 46-kDa species binds to concanavalin A-Sepharose, whereas the 44-kDa species does not (Fig. 3). Neither of the two proteins bound to *R. communis* agglutinin-agarose, which recognizes terminal or sialylated Galβ1–4GlcNAc sequences (Baenzinger and Fiete, 1979). Therefore, the 46-kDa species may be produced by *N*-glycosylation (high-mannose type) of the 44-kDa species at residue 47. To confirm that the cDNA codes the 44-kDa protein, the cDNA was cloned into an expression vector (pCAGGSneo) and introduced into COS cells. Upon immunoblot analysis, COS cells transfected with the cDNA in a sense orientation showed a 44-kDa band and a faint 46-kDa band, whereas COS cells transfected with the cDNA in an antisense orientation gave faint bands in the region (Fig. 4). We consider that the faint bands (46 and 44 kDa) represent endogenous reticulocalbin in COS cells. Even though the 44-kDa band is present in COS cells transfected with antisense cDNA, the intensity of the band greatly increased in COS cells transfected with sense cDNA. Therefore, we conclude

FIG. 2. **Nucleotide sequence of reticulocalbin cDNA and predicted amino acid sequence of protein.** Nucleotides are numbered on the *right*. A signal sequence is denoted by the *underline*. A consensus *N*-glycosylation site is marked with a *filled triangle*. Residues corresponding to the calcium-binding loop of the EF-hands predicted from the homology of this family of calcium-binding proteins and the polyadenylation signals are *underlined*. In clones O9 and O32, which have a shorter 3′-flanking sequence, the poly(A) tails start at positions 1589 and 1744, respectively.



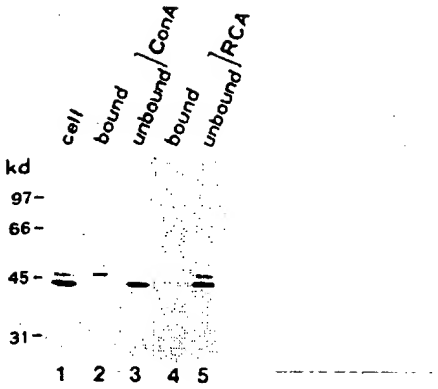


FIG. 3. **Immunoblot analysis of reticulocalbin.** PYS-2 cells were boiled in SDS-PAGE sample buffer (*lane 1*) or were lysed with Nonidet P-40 and fractionated on ConA-Sepharose (*lanes 2* and *3*) or *R. communis* agglutinin (*RCA*)-agarose (*lanes 4* and *5*). Bound materials specifically eluted with the respective haptenic sugars (*lanes 2* and *4*), and unbound materials (*lanes 3* and *5*) were immunoblotted with anti-reticulocalbin antibodies.

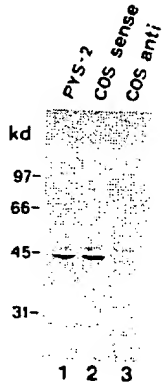


FIG. 4. **Immunoblots of reticulocalbin transiently expressed in COS cells.** PYS-2 (positive control) (*lane 1*) and COS cells transfected with reticulocalbin cDNA in an expression vector in a sense (*lane 2*) or antisense (*lane 3*) orientation were boiled in SDS-PAGE sample buffer and immunoblotted with anti-reticulocalbin antibodies. The antibodies recognized endogenous COS cell reticulocalbin in the immunoblots, but not in immunofluorescence and immunoprecipitation (compare with Figs. 8 and 9); probably the conformation-dependent epitopes detectable in these assays are species-specific.

that the cDNA clone has the entire sequence encoding the 44-kDa protein.

Reticulocalbin Is a Ca^{2+}-binding Protein with EF-hand Motifs—Analysis of the cDNA sequence shows that the predicted protein contains six repeats of ~30 amino acids (Fig. 5). Each repeat has the general feature of a high affinity Ca^{2+}-binding EF-hand domain according to Kretsinger's rule (Kretsinger, 1980). Comparing the amino acid sequence of these domains with those required for a perfect EF-hand Ca^{2+}-binding site, there is a varying degree of divergence. The 5 oxygen-containing residues important for the coordination of Ca^{2+} are present in all the predicted sites. The central glycine is conserved in sites I, IV, and V, whereas in sites II, III, and VI, it is replaced either by glutamic acid or leucine. The replacements appear not to be cloning artifacts because the independent cDNA clones have the same sequence in the region. Furthermore, the sequence of the corresponding region of genomic DNA clones also has the same sequence.[2] The secondary structure

[2] M. Ozawa, unpublished results.

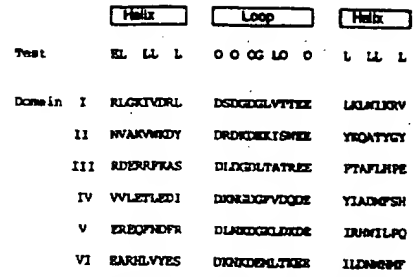


FIG. 5. **Calcium-binding domains of reticulocalbin.** The protein sequence (Fig. 2) was analyzed for homologies to the test sequence for a Ca^{2+}-binding EF-hand motif. The amino acid sequence is shown in one-letter code. In the test sequence, *L* represents hydrophobic residues, and *O* represents oxygen-containing residues.

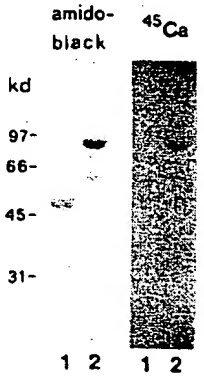


FIG. 6. **Calcium blots of recombinant reticulocalbin.** Maltose-binding protein (2 μg) (*lanes 1*) or a recombinant reticulocalbin of the maltose-binding protein fusion (2 μg) (*lanes 2*) purified on amylose resin was electrophoresed and transferred to a nitrocellulose membrane. After incubation with $^{45}Ca^{2+}$, the membrane was exposed to x-ray film. The same membrane was stained with Amido Black to detect proteins. The apparent molecular mass (88 kDa) of the fusion protein is in good agreement with the expected size (86 kDa).

prediction (Chou and Fasman, 1978) shows that the replacement of glycine with glutamic acid in sites II and VI causes the formation of an α-helix at the sites instead of a loop structure. Therefore, sites II and VI of reticulocalbin probably no longer bind Ca^{2+}. Although the glycine is replaced by leucine, site III appears capable of forming the loop structure. The replacement of the normally conserved glycine with lysine has been reported in the EF-hands of the fibrinogen γ-chain (Dang *et al.*, 1985) and secreted protein acidic and rich in cysteine (BM-40, osteonectin) (Engel *et al.*, 1987). Therefore, site III seems to bind Ca^{2+}.

To test whether reticulocalbin actually binds Ca^{2+}, recombinant reticulocalbin was analyzed by $^{45}Ca^{2+}$ blotting. The cDNA coding mature reticulocalbin was cloned into the MBP fusion vector (pMAL-c), and the recombinant protein was expressed in *E. coli* as a fusion protein with MBP. After purification by affinity chromatography on a column containing amylose resin, the fusion protein was electrophoresed on a gel, transferred to a nitrocellulose membrane, and incubated with Ca^{2+}. MBP alone did not bind Ca^{2+}; however, the fusion protein did (Fig. 6).

Reticulocalbin Is a Luminal ER Protein—After homogenization of PYS-2 cells, reticulocalbin remained in the low speed (5000 × *g*) supernatant, but sedimented together with crude membranes during high speed (100,000 × *g*) centrifugation (Fig. 7*A*). It was solubilized from the membrane by Triton X-100 or by sonication in the absence of detergents (Fig. 7*A*). Reticulocalbin partitioned into the aqueous phase when cells

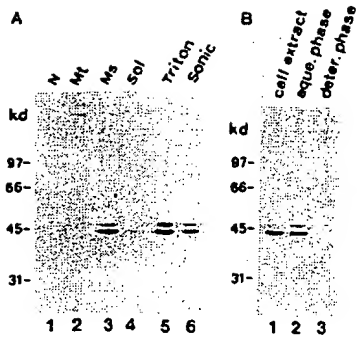


FIG. 7. **Reticulocalbin is a microsomal protein with a hydrophilic nature.** *A*, PYS-2 cells were homogenized and fractionated by differential centrifugation into nuclear (*N*; *lane 1*), mitochondrial (*Mt*; *lane 2*), microsomal (*Ms*; *lane 3*), and soluble (*Sol*; *lane 4*) fractions and immunoblotted. Reticulocalbin in the microsomal fraction was solubilized with Triton X-100 (*lane 5*) or released by sonication (*Sonic*; *lane 6*). *B*, cells were extracted with Triton X-114, and the extract (*lane 1*) was analyzed by phase separation. Reticulocalbin was found in the aqueous (*aque.*) phase (*lane 2*), and not in the detergent (*deter.*) phase (*lane 3*).

were extracted with Triton X-114 and phase-separated (Fig. 7*B*).

To localize reticulocalbin, PYS-2 cells were stained with anti-reticulocalbin antibodies by indirect immunofluorescence. When the cells were fixed with formaldehyde and permeabilized with Triton X-100, the intracellular ER regions (but not the cell surface) were stained (Fig. 8*A*). Fig. 8*A* shows that reticulocalbin is localized in the perinuclear system of membranes corresponding to that of the ER of PYS-2 cells. Since the ER seemed not to be well developed in PYS-2 cells, we stained transfected COS cells expressing reticulocalbin. Untransfected cells were not detectably stained with the antibody (data not shown). All cells expressing reticulocalbin exhibited prominent staining of the perinuclear region as well as a lattice of fine tubular structures (Fig. 8*C*), but there was no obvious staining of the Golgi apparatus. This pattern is typical of that obtained for proteins that are retained in the ER (Munro and Pelham, 1987). Furthermore, the structure that contained reticulocalbin also stained with concanavalin A (Fig. 8*D*), which predominantly stains ER structures (Tartakoff and Vassalli, 1983), confirming that they represented the ER. Because of the bright staining in the perinuclear region, however, it was difficult to get a clearly visible pattern of the tubule network. We overcame this issue using a confocal scanning laser microscope. Fig. 8*E* shows the distribution of reticulocalbin in the transfected COS cells. The image confirms the results obtained using conventional microscopy and allows detailed examination of the distribution of the protein. There is an intense reticular staining of the ER.

Reticulocalbin has a putative amino-terminal signal peptide, but no hydrophobic transmembrane segment, which is consistent with transfer of the entire protein into the lumen of the ER. To verify that the amino-terminal stretch of hydrophobic amino acids functions as a signal sequence, we carried out the experiments described below. The rationale is as follows. Luminal ER proteins in animal cells are prevented from being secreted by a sorting system that recognizes the carboxyl-terminal sequence KDEL (Munro and Pelham, 1987). Instead of KDEL at the carboxyl terminus, reticulocalbin has the closely related sequence HDEL. Although the HDEL sequence has been reported to be inefficient as an ER retention signal in animal cells because a lysozyme fusion protein with the HDEL sequence at the carboxyl terminus was efficiently secreted into the medium (Pelham *et al.*, 1988),

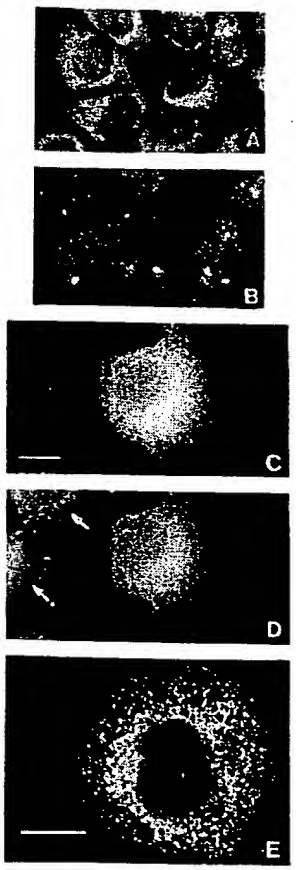


FIG. 8. **Localization of reticulocalbin by immunofluorescent staining.** PYS-2 cells (*A* and *B*) or COS cells transfected with reticulocalbin cDNA (*C–E*) were stained with anti-reticulocalbin antibodies (*A*, *C*, and *E*) or control antibodies (*B*). In *C* and *D*, cells were double-labeled with anti-reticulocalbin antibody and biotinylated ConA followed by fluorescein-labeled goat anti-rabbit antibody and rhodamine-labeled avidin, and distribution of reticulocalbin (*C*) and ConA (*D*) was visualized. *Arrows* in *D* shows cells negative for reticulocalbin staining (*C*). *A–D*, cells were photographed using an immunofluorescence microscope at the same magnification. *Bar*, 20 μm. *E*, an image generated by an MRC500 confocal scanning laser microscope on a video monitor. *Bar*, 25 μm. The microscope was focused on the lower part of the cell.

recent experiments on liver carboxylesterases have demonstrated that the sequence is functional at least in this case (Robbi and Beaufay, 1991). Therefore, the removal of the carboxyl-terminal HDEL sequence could allow the mutant protein to escape from the retrieval machinery and to be secreted into the medium if the amino-terminal sequence is the signal sequence. For this, a mutant protein specifically lacking the HDEL sequence was constructed. The synthesis and secretion of the wild-type and mutant reticulocalbins were evaluated by transient expression in COS cells (Fig. 9). After labeling with [^{35}S]methionine for 30 min and a chase (3 h) with excess unlabeled methionine, proteins were immunoprecipitated from the cell lysates and from media and then analyzed by SDS-PAGE. Under these conditions, wild-type reticulocalbin was secreted very slowly from the COS cells. Less than 10% of the pulse-labeled protein was found in the medium. Reticulocalbin lacking the HDEL sequence, however, was secreted rapidly from the COS cells (Fig. 9). Densitometry of the fluorographs showed that ~80% of the mutant protein was recovered from the medium. No reticulocalbin was secreted from PYS-2 cells under the same conditions (data not shown). These results show that the amino-terminal

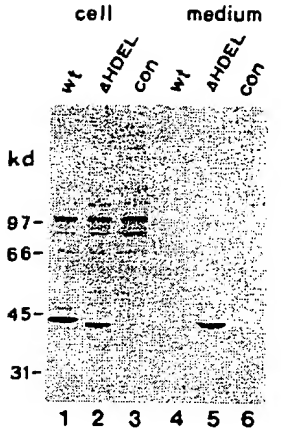


FIG. 9. **Immunoprecipitation of wild-type reticulocalbin and mutant reticulocalbin lacking carboxyl-terminal HDEL peptide.** COS cells transfected with wild-type (*wt*) (*lanes 1* and *4*) or mutant (*ΔHDEL*) (*lanes 2* and *5*) reticulocalbin cDNA in an expression vector or the vector without the cDNA (control (*con*)) (*lanes 3* and *6*) were labeled with [^{35}S]methionine for 30 min. After a chase for 2 h with excess unlabeled methionine, the cells and media were collected. The cell lysates (*lanes 1–3*) and media (*lanes 4–6*) were incubated with anti-reticulocalbin antibodies, and immunocomplexes were analyzed by SDS-PAGE.

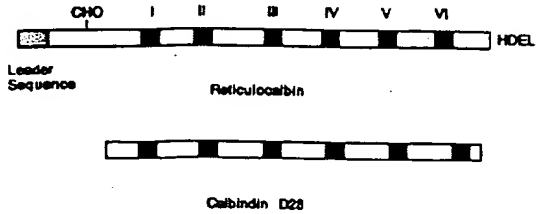


FIG. 10. **Domain structure of reticulocalbin and calbindin D28.** The *filled boxes* represent the sequences corresponding to the loop of the EF-hand structure. The consensus *N*-glycosylation site is marked by *CHO*.

hydrophobic amino acids of reticulocalbin function as a signal for the transfer of the entire protein into the lumen of the ER.

DISCUSSION

We identified and characterized a novel ER resident Ca^{2+}-binding protein called reticulocalbin by cDNA cloning, sequence analysis, and biochemical as well as cell biological studies. The major structural features of reticulocalbin, as deduced from the cDNA sequence, are outlined in Fig. 10. The protein consists of 325 amino acids with a single hydrophobic sequence at the amino terminus that constitutes a leader sequence. At the amino-terminal region of the mature protein, there is a potential *N*-glycosylation site, which was indeed partially glycosylated. The carboxyl terminus contains a version of the KDEL ER retention signal, HDEL. The rest of the protein consists of the six domains of the EF-hand motif of high affinity Ca^{2+}-binding proteins.

The most interesting feature of the sequence of reticulocalbin is the presence of the domains of the EF-hand motif. Therefore, reticulocalbin can be classified into the EF-hand calcium-binding protein superfamily, which includes calmodulin, troponin C, and myosin light chain. All members of this diverse protein family share multiple conserved sequence domains based on a distinct helix-loop-helix structure, the EF-hand (Kretsinger, 1980). The loop constitutes the Ca^{2+}-binding site. The proteins of the superfamily identified to date contain from two to eight EF-hands r variants thereof. In some of them, the EF-hands have been duplicated or lost; and in others, the calcium binding properties have been altered or lost entirely (Heizman and Hunziker, 1991).

Although the reticulocalbin sequence has no significant homology to any other proteins except for the EF-hand motifs, the overall structure of reticulocalbin is similar to that of calbindin D28 and calretinin (Rogers, 1989) in that both proteins have six EF-hand motif domains. Calbindin D28 and calretinin have been found at high concentrations in the central and peripheral nervous systems of many species, but their function is presently unknown. Calbindin D28 binds only four Ca^{2+} atoms/mol of protein, and the second and sixth domains may have lost their Ca^{2+} binding capability because some oxygen-containing amino acids in the loop are missing (Hunziker, 1986). Similarly, the second and sixth domains of reticulocalbin seem to have lost their Ca^{2+} binding ability. Reticulocalbin, however, has a long amino-terminal extension as well as a short carboxyl-terminal extension. The latter has an HDEL sequence that could serve as part of the retention signal for the protein in the ER. The amino-terminal extension is composed of a leader sequence, which directs the protein to be translocated into the lumen of the ER, and the amino-terminal region of the mature protein of ~50 amino acids, where a single *N*-glycosylation site resides.

The evidence presented here demonstrates that reticulocalbin is a luminal Ca^{2+}-binding protein residing in the ER. The sequence of the cDNA clone showed that it has a secretory leader peptide that is not present on the mature protein. This leader sequence is functional in that it causes reticulocalbin to enter the ER, as shown by its glycosylation, and to be secreted into the medium since a mutant reticulocalbin lacking the carboxyl-terminal HDEL sequence was secreted in COS cells. Mature reticulocalbin does not contain a hydrophobic transmembrane sequence, and there is no evidence for its secretion from the cells; it thus appears likely that it accumulates as a soluble protein within the intracellular membrane system. Consistent with this is the finding that reticulocalbin cosediments with crude membranes (microsomes) and can be released from the membranes with detergents or by sonication. Upon phase separation in Triton X-114, reticulocalbin partitioned into the aqueous phase. Furthermore, anti-reticulocalbin antibody stained endogenous reticulocalbin in PYS-2 cells as well as transiently expressed reticulocalbin in COS cells with an ER-like pattern.

The carboxyl-terminal HDEL tetrapeptide, a variant of the KDEL ER retention signal, seems to be part of a signal that prevents it from being secreted from the cell since the mutant reticulocalbin lacking HDEL is secreted. After identifying the carboxyl-terminal KDEL sequence as an ER retention signal, a number of variants of this sequence have been reported (Pelham, 1990), including sequences KEEL for the protein ERp72 (Mazzarella *et al.*, 1990) and RDEL in a 55-kDa thyroid hormone-binding protein (Fliegel *et al.*, 1990). The retrieval system in the yeast *Saccharomyces cerevisiae* recognizes the carboxyl-terminal HDEL sequence of ER resident proteins (Pelham *et al.*, 1988). The HDEL sequence, however, is reportedly inefficient as an ER retention signal in animal cells because addition of the sequence to a lysozyme fusion protein did not cause its retention in the ER when the protein was expressed in COS cells (Pelham *et al.*, 1988). Despite this observation, HDEL can be used as the ER retention signal in some ER resident proteins such as reticulocalbin because the efficiency of retention can vary, depending on the proteins to be analyzed, as has been reported (Zagouras and Rose, 1989). Recently, HDEL in carboxylesterase has been shown to be

functional in animal cells (Robbi and Beaufay, 1991). The other possibility, that the removal of HDEL caused altered protein folding, which in turn affected the interaction of reticulocalbin with other ER resident proteins, however, is not formally excluded.

Although our studies revealed valuable information about the structure and localization of reticulocalbin, the function of this protein remains unknown. Its localization in the lumen of the ER and its expression in different types of cells suggest a role in protein synthesis, modification, and intracellular transport. We speculate that reticulocalbin functions in the regulation of Ca^{2+}-dependent activities in the lumen of the ER or post-ER compartment. One intriguing possibility is that reticulocalbin is involved in the retention mechanism of KDEL-terminated proteins in the ER, in which Ca^{2+} may be involved (Booth and Koch, 1989; Kelly, 1990). Reticulocalbin may associate with an as yet unidentified protein and regulate its activity by binding Ca^{2+}. That reticulocalbin may perform multiple functions like calmodulin should be considered.

Finally, we believe that the name reticulocalbin is appropriate for the protein because it reflects its intracellular localization in the lumen of the ER (reticuloplasm), established Ca^{2+} binding properties, and the six calbindin D28-like domains of the EF-hand motifs.

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Munro, S., and Pelham, H. R. B. (1986) *Cell* **46**, 291–300
Munro, S., and Pelham, H. R. B. (1987) *Cell* **48**, 899–907
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Sorger, P., and Pelham, H. R. B. (1987) *J. Mol. Biol.* **194**, 341–344
Strynadka, N. C. J., and James, M. N. G. (1989) *Annu. Rev. Biochem.* **58**, 951–998
Tartakoff, A. M., and Vassalli, P. (1983) *J. Cell Biol.* **97**, 1243–1248
Zagouras, P., and Rose, J. K. (1989) *J. Cell Biol.* **109**, 2633–2640

SeqServer
biology *in silico*

ClustalW Results

Sequences | Help

Retrieval | BLAST2 | FASTA | ClustalW | GCG Assembly | Phrap | Translation

- 1601793CD1
- g220582

CLUSTAL W (1.7) Multiple Sequence Alignments

```
Sequence format is Pearson
Sequence 1: 1601793CD1      315 aa
Sequence 2: g220582         325 aa
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  58
Start of Multiple Alignment
There are 1 groups
Aligning...
Group 1: Sequences:   2      Score:3364
Alignment Score 1226
CLUSTAL-Alignment file created  [baax0ayYk.aln]
CLUSTAL W (1.7) multiple sequence alignment


1601793CD1      -MDLRQFLMCLSLCTAFALS---KPTEKKDRVHH-EPQLSDKVHNDAQSFDYDHDAFLGA
g220582         MARGGRLGLALGLLLALVLALRAKPTVRKERVVRPDSELGERPPEDNQSFQYDHEAFLGK
                     :: :.*.*  *:.*:   *** :*:** : :.:*.::  :* ***:***:****

1601793CD1      EEAKTFDQLTPEESKERLGKIVSKIDGDKDGFVTVDELKDWIKFAQKRWIYEDVERQWKG
g220582         EDSKTFDQLSPDESKERLGKIVDRIDSDGDGLVTTEELKLWIKRVQKRYIYDNVAKVWKD
                *:::******:*:**********.:**.* **:**.:*** *** .***:**::* : **.

1601793CD1      HDLNEDGLVSWEEYKNATYGYVLDDP----DPDDGFNYKQMMVRDERRFKMADKDGDLIA
g220582         YDRDKDEKISWEEYKQATYGYYLGNPAEFHDSSDHHTFKKMLPRDERRFKASDLDGDLTA
                :* ::*  :******:***** *.:*    *..* ..:*:*: ******* :* **** *

1601793CD1      TKEEFTAFLHPEEYDYMKDIVVQETMEDIDKNADGFIDLEEYIGDMYSHDGNTDEPEWVK
g220582         TREEFTAFLHPEEFEHMKEIVVLETLEDIDKNGDGFVDQDEYIADMFSHEDNGPEPDWVL
                *:***********:::**:*** **:******.***:* :***.**:**:.*  **:**

1601793CD1      TEREQFVEFRDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIV
g220582         SEREQFNDFRDLNKDGKLDKDEIRHWILPQDYDHAQAEARHLVYESDKNKDEMLTKEEIL
                :***** :*** *:***:**:* :.****.*****:***********:***  ******:

1601793CD1      DKYDLFVGSQATDFGEALVR-HDEF
g220582         DNWNMFVGSQATNYGEDLTKNHDEL
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Sequence Revision History

Search for Genes

Entrez Nucleotide

Revision history for "D13003"			
GI	**Version**	**Update Date**	**Status**
220581	1	Jul 25 2002 14:17	Live
220581	1	Sep 8 1999 19:10	Dead
220581	1	Mar 17 1999 21:53	Dead
220581	1	Jun 5 1997 14:48	Dead
220581	1	Apr 29 1993 10:48	Dead

Accession D13003 was first seen at NCBI on Apr 29 1993 10:48

Related resources

1: BAA02366. reticulocalbin [M...[gi:220582]

BLink, Domains, Links

```
LOCUS       BAA02366                 325 aa            linear   ROD 09-SEP-1999
DEFINITION  reticulocalbin [Mus musculus].
ACCESSION   BAA02366
VERSION     BAA02366.1  GI:220582
DBSOURCE    locus MUSRCAL accession D13003.1
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (residues 1 to 325)
  AUTHORS   Ozawa,M. and Muramatsu,T.
  TITLE     Reticulocalbin, a novel endoplasmic reticulum resident
            Ca(2+)-binding protein with multiple EF-hand motifs and a
            carboxyl-terminal HDEL sequence
  JOURNAL   J. Biol. Chem. 268 (1), 699-705 (1993)
  MEDLINE   93107083
   PUBMED   8416973
REFERENCE   2  (residues 1 to 325)
  AUTHORS   Ozawa,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-AUG-1992) Masayuki Ozawa, Faculty of Medicine,
            Kagoshima University, Department of Biochemistry; 8-35-1
            Sakuragaoka, Kagoshima, Kagoshima 890, Japan (Tel:0992-75-5246,
            Fax:0992-64-5618)
FEATURES             Location/Qualifiers
     source          1..325
                     /organism="Mus musculus"
                     /strain="129/Sv"
                     /db_xref="taxon:10090"
                     /clone="O1 and M10"
                     /cell_line="OTT6050"
                     /cell_type="teratocarcinoma"
     Protein         1..325
                     /product="reticulocalbin"
     sig_peptide     1..20
     mat_peptide     21..325
                     /product="unnamed"
     CDS             1..325
                     /gene="Rcal"
                     /coded_by="D13003.1:35..1012"
ORIGIN
        1 marggrlgla lglllalvla lrakptvrke rvvrpdselg erppednqsf qydheaflgk
       61 edsktfdqls pdeskerlgk ivdridsdgd glvtteelkl wikrvqkryi ydnvakvwkd
      121 ydrdkdekis weeykqatyg yylgnpaefh dssdhhtfkk mlprderrfk asdldgdlta
      181 treeftaflh peefehmkei vvletledid kngdgfvdqd eyiadmfshe dngpepdwvl
      241 sereqfndfr dlnkdgkldk deirhwilpq dydhaqaear hlvyesdknk demltkeeil
      301 dnwnmfvgsq atnygedltk nhdel
//
```

```
Qy     181 HPEEYDYMKDIVVQETMEDIDKNADGFIDLEEYIGDMYSHDGNTDEPEWVKTEREQFVEF 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     181 HPEEYDYMKDIVVQETMEDIDKNADGFIDLEEYIGDMYSHDGNTDEPEWVKTEREQFVEF 240

Qy     241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGS 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGS 300

Qy     301 QATDFGEALVRHDEF 315
           |||||||||||||||
Db     301 QATDFGEALVRHDEF 315


RESULT 2
O35783
ID   O35783      PRELIMINARY;      PRT;   315 AA.
AC   O35783;
DT   01-JAN-1998 (TrEMBLrel. 05, Created)
DT   01-JAN-1998 (TrEMBLrel. 05, Last sequence update)
DT   01-MAR-2002 (TrEMBLrel. 20, Last annotation update)
DE   CBP-50 protein.
OS   Rattus norvegicus (Rat).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Rattus.
OX   NCBI_TaxID=10116;
RN   [1]
RP   SEQUENCE FROM N.A.
RC   STRAIN=SPRAGUE-DAWLEY; TISSUE=BRAIN;
RA   Hseu M.J.;
RL   Submitted (OCT-1997) to the EMBL/GenBank/DDBJ databases.
RN   [2]
RP   SEQUENCE FROM N.A.
RC   STRAIN=SPRAGUE-DAWLEY; TISSUE=BRAIN;
RX   MEDLINE=93107083; PubMed=8416973;
RA   Ozawa M., Muramatsu T.;
RT   "Reticulocalbin: a novel endoplasmic reticulum resident calcium-
RT   binding protein with multiple EF-hand motifs and a caboxyl-terminal
RT   HDEL sequence.";
RL   J. Biol. Chem. 268:699-705(1993).
DR   EMBL; AJ001929; CAA05100.1; -.
DR   InterPro; IPR002048; EF-hand.
DR   Pfam; PF00036; efhand; 6.
DR   SMART; SM00054; EFh; 2.
DR   PROSITE; PS00018; EF_HAND; UNKNOWN_4.
SQ   SEQUENCE   315 AA;  36996 MW;  06B7FED579929E98 CRC64;

  Query Match             89.1%;  Score 1516;  DB 11;  Length 315;
  Best Local Similarity   88.6%;  Pred. No. 8.3e-89;
  Matches  279;  Conservative   19;  Mismatches   17;  Indels    0;  Gaps    0;

Qy       1 MDLRQFLMCLSLCTAFALSKPTEKKDRVHHEPQLSDKVHNDAQSFDYDHDAFLGAEEAKT 60
           ||||||||||||||||||||||||||||||||||||||||:|||||||||||||||:
Db       1 MDLRQFLMCLSLCTAFALSKPTEKKDRVHHEPQLSDKVHNDAQNFDYDHDAFLGAEEAKS 60

Qy      61 FDQLTPEESKERLGKIVSKIDGDKDGFVTVDELKDWIKFAQKRWIYEDVERQWKGHDLNE 120
           | |||||||||:|| || ||| |||||||  |||  || |||::||::|| ||:  |:|:
Db      61 FGQLTPEESKEKLGMIVDKIDTDKDGFVTEGELKSRIKHAQKKYIYDNVENQWQEFDMNQ 120

Qy     121 DGLVSWEEYKNATYGYVLDDPDPDDGFNYKQMMVRDERRFKMADKDGDLIATKEEFTAFL 180
           |||:||:||:| |||  |||||||||||| :|||||||||||:||||||||||||||||
Db     121 DGLISWDEYRNVTYGTYLDDPDPDDGFNYKPIMVRDERRFKMADQDGDLIATKEEFTAFL 180

Qy     181 HPEEYDYMKDIVVQETMEDIDKNADGFIDLEEYIGDMYSHDGNTDEPEWVKTEREQFVEF 240
           ||||||||||:||||||||:|||||||||||||||||||||| |||:||||||||||||||
Db     181 HPEEYDYMKDIVLQETMEDIDQNADGFIDLEEYIGDMYSHDGNADEPQWVKTEREQFVEF 240

Qy     241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGS 300
           ||||||||||||||||||||||||||||||||||||:|||||||||||||||||||||||||
Db     241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQDKDGKLTKEEIVDKYDLFVGS 300

Qy     301 QATDFGEALVRHDEF 315
           |||||||||||||||
Db     301 QATDFGEALVRHDEF 315


RESULT 3
Q9NR43
ID   Q9NR43      PRELIMINARY;      PRT;   296 AA.
AC   Q9NR43;
DT   01-OCT-2000 (TrEMBLrel. 15, Created)
DT   01-OCT-2000 (TrEMBLrel. 15, Last sequence update)
DT   01-JUN-2002 (TrEMBLrel. 21, Last annotation update)
DE   Crocalbin-like protein (Fragment).
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
OX   NCBI_TaxID=9606;
RN   [1]
RP   SEQUENCE FROM N.A.
RC   TISSUE=BRAIN;
RX   MEDLINE=99192326; PubMed=10094503;
RA   Hseu M.J., Yen C.H., Tzeng M.C.;
RT   "Crocalbin: a new calcium-binding protein that is also a binding
RT   protein for crotoxin, a neurotoxic phospholipase A2.";
RL   FEBS Lett. 445:440-444(1999).
RN   [2]
RP   SEQUENCE FROM N.A.
RC   TISSUE=BRAIN;
RA   Hseu M.J., Tzeng M.C.;
RL   Submitted (APR-2000) to the EMBL/GenBank/DDBJ databases.
DR   EMBL; AF257659; AAF76141.1; -.
DR   InterPro; IPR002048; EF-hand.
DR   Pfam; PF00036; efhand; 6.
DR   SMART; SM00054; EFh; 3.
DR   PROSITE; PS00018; EF_HAND; UNKNOWN_4.
FT   NON_TER       1       1
SQ   SEQUENCE   296 AA;  34990 MW;  D011D029ADE2A02E CRC64;

  Query Match             87.7%;  Score 1491;  DB 4;  Length 296;
  Best Local Similarity   92.6%;  Pred. No. 3e-87;
  Matches  274;  Conservative    9;  Mismatches   13;  Indels    0;  Gaps    0;

Qy      20 KPTEKKDRVHHEPQLSDKVHNDAQSFDYDHDAFLGAEEAKTFDQLTPEESKERLGKIVSK 79
           ||||||||||||||||||||||||||||||||||||||||||||||||||||| || |
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Qy      80 IDGDKDGFVTVDELKDWIKFAQKRWIYEDVERQWKGHDLNEDGLVSWEEYKNATYGYVLD 139
           || ||||||| ||| ||| |||::||:||| ||:  |:|:|||:||:||:| ||| ||
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           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Qy     200 IDKNADGFIDLEEYIGDMYSHDGNTDEPEWVKTEREQFVEFRDKNRDGKMDKEETKDWIL 259
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Qy     260 PSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGSQATDFGEALVRHDEF 315
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     241 PSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGSQATDFGEALVRHDEF 296


RESULT 4
Q9MZ46
ID   Q9MZ46      PRELIMINARY;      PRT;   296 AA.
AC   Q9MZ46;
DT   01-OCT-2000 (TrEMBLrel. 15, Created)
DT   01-OCT-2000 (TrEMBLrel. 15, Last sequence update)
DT   01-MAR-2002 (TrEMBLrel. 20, Last annotation update)
DE   Crocalbin-like protein (Fragment).
OS   Sus scrofa (Pig).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Cetartiodactyla; Suina; Suidae; Sus.
OX   NCBI_TaxID=9823;
RN   [1]
```

Docket No.: PF-0358-2 DIV

Response Under 37 C.F.R. 1.116 - Expedited Procedure
Examining Group 1653

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By: _____ Printed: JEANNIE G. LABRA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of: Bandman et al.

Title:	HUMAN RETICULOCALBIN ISOFORMS		
Serial No.:	09/847,809	Filing Date:	May 01, 2001
Examiner:	Carlson, K.	Group Art Unit:	1653

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

BRIEF ON APPEAL

Sir:

Further to the Notice of Appeal filed November 6, 2003, and received by the USPTO on November 10, 2003, herewith are three copies of Appellants' Brief on Appeal. Appellants hereby request a 1-month extension of time in order to file this Brief. Authorized fees include the statutory fee of $110.00 for a 1-month extension of time, as well as the $ 330.00 fee for the filing of this Brief.

This is an appeal from the decision of the Examiner finally rejecting claims 10, 30, 31, 33, 36, 37, and 39-42 of the above-identified application.

(1) REAL PARTY IN INTEREST

The above-identified application is assigned of record to Incyte Pharmaceuticals, Inc. (now Incyte Corporation, formerly known as Incyte Genomics, Inc.) (Reel 8922, Frame 0082), which is the real party in interest herein.

(2) RELATED APPEALS AND INTERFERENCES

Appellants, their legal representative and the assignee are not aware of any related appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the instant appeal.

(3) STATUS OF THE CLAIMS

Claims rejected:	Claims 10, 30, 31, 33, 36, 37, and 39-42
Claims allowed:	None
Claims canceled:	Claims 1-9, 11-28 and 45-48
Claims withdrawn:	Claims 29, 32, 34, 35, 38 and 43-44
Claims on Appeal:	Claims 10, 30, 31, 33, 36, 37, and 39-42 (A copy of the claims on appeal can be found in the attached Appendix).

(4) STATUS OF AMENDMENTS AFTER FINAL

There were no amendments submitted after Final Rejection;

(5) SUMMARY OF THE INVENTION

Appellants' invention is directed, *inter alia,* to isolated antibodies which specifically bind to human reticulocalbin isoform RCNδ, a polypeptide comprising the amino acid sequence of SEQ ID NO:3. Nucleic acids encoding the RCNδ of the present invention were first identified in Incyte Clone 1601793 from the bladder cDNA library (BLADNOT03) using a computer search for amino acid sequence alignments (Specification at page 17, lines 15-17). The amino acid sequence of SEQ ID NO:3, as shown in Figures 2A, 2B, 2C, 2D, 2E, 2F, and 2G [of the Specification]...is 315 amino acids in length, has an N-terminal signal peptide (M-1 to S-19), and has seven EF-hands (Specification at

page 17, lines 22-25). As shown in Figures 4A and 4B [of the Specification], RCNδ has chemical and structural homology with human reticulocalbin (GI 1262329; SEQ ID NO:5). In particular, RCNδ and human reticulocalbin share 58% identity, share an N-terminal signal peptide, and six EF-hands. As illustrated by Figures 5B and 5C [of the Specification], RCNδ and human reticulocalbin have rather similar hydrophobicity plots. Northern analysis shows the expression of this sequence in various libraries, at least 50% of which are immortalized or cancerous, at least 22% of which involve immune response, and at least 19% of which involve fetal or proliferating tissue. Of particular note is the expression of RCNδ in heart, gut, prostate, and smooth muscle; and at sites of hematopoiesis (Specification at page 17, line 29 to page 18, line 7). Overexpression of reticulocalbin mRNA has been associated with the increased matrigel invasive properties of three human breast cancer cell lines. Conversely, reticulocalbin was not found to be expressed in two poorly invasive breast cancer cell lines (Liu, Z. et al. (1997) Biochem. Biophys. Res. Comm. 231:283-289). (Specification at page 3, lines 8-11.) The antibodies, compositions and methods of the present invention are useful for, *inter alia*, disease detection and expression profiling as well as in toxicological testing applications.

(6) ISSUES

1. Whether claims 10, 30, 31, 33, 36, 37 and 39-42 directed to isolated antibodies which specifically bind to SEQ ID NO:3 meet the novelty requirement of 35 U.S.C. §102(a).

2. Whether claims 10, 30, 31, 33, 36, 37 and 39-42 directed to isolated antibodies which specifically bind to SEQ ID NO:3 meet the novelty requirement of 35 U.S.C. §102(b).

3. Whether claims 36 and 39 directed to isolated antibodies which specifically bind to SEQ ID NO:3, produced by the methods of withdrawn claims 35 and 38, meet the definiteness requirement of 35 U.S.C. §112, second paragraph.

(7) GROUPING OF THE CLAIMS

As to Issue 1

All of the claims on appeal are grouped together.

As to Issue 2

All of the claims on appeal are grouped together.

As to Issue 3

Claims 36 and 39 are grouped together.

(8) APPELLANTS' ARGUMENTS

Issue 1--Claim rejections under 35 U.S.C. § 102(a)

Claims 10, 30, 31, 33, 36, 37 and 39-42 have been rejected under 35 U.S.C. § 102(a) as allegedly being anticipated by Yabe et al. (July 18, 1997; J. Biol. Chem. 272:18323-18239; of record, copy supplied for the convenience of the Board as Attachment 1). Appellants respectfully disagree with the Examiner's analysis of the art in Yabe et al. and submit that claims 10, 30, 31, 33, 36, 37 and 39-42 are not anticipated under 35 U.S.C. § 102(a) by this publication.

For a reference to anticipate claimed subject matter under any section of 35 U.S.C. § 102, "every element of the claimed invention must be identically shown in a single reference." *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990). Furthermore, the MPEP provides guidance for interpretation of the case law that has been generated with regard to 35 U.S.C. § 102: "the reference ***must teach every aspect of the claimed invention either explicitly or impliedly***. Any feature not directly taught must be inherently present" M.P.E.P. § 706.02 (emphasis added). Moreover, M.P.E.P. § 2112 states that "[t]he fact that a certain result or characteristic **may** occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic" (emphasis in original). M.P.E.P § 2112 further states that "[t]he examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic **necessarily** flows from the teachings of the applied prior art" (emphasis in original). Yet the Final Office Action provides no such basis or reasoning. Furthermore, the Examiner has attempted to support this rejection with articles by Bost et al., and Bendayan (of record; copies supplied for the convenience of the Board

as Attachments 2 and 3 respectively), Abaza et al. (of record; copy supplied for the convenience of the Board as Attachment 4) and Li et al. (of record; copy supplied for the convenience of the Board as Attachment 5); and with U.S. Pat. No. 6,210,670 (Berg; of record; copy supplied for the convenience of the Board as Attachment 6). Appellants strongly disagree with the Examiner's position for the following reasons.

I. Claims 10, 30, 31, 33, 36, 37 and 39-42 are not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

Claim 10 recites "An isolated antibody which ***specifically binds*** to a polypeptide comprising the amino acid sequence of SEQ ID NO:3." (Emphasis added.) In order to anticipate the claimed subject matter under any section of 35 U.S.C. § 102, "the reference ***must teach every aspect of the claimed invention either explicitly or impliedly***" M.P.E.P. § 706.02 (emphasis added). Under these parameters, the claimed subject matter is not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

It appears that the basis of this rejection is the Examiner's assertion that "Yabe et al....teach calumenin having 98.2% identity to SEQ ID NO:3. On page 18234, col. 1, para. 3, Yabe et al. made antibodies against calumenin, anti-protein-disulfide isomerase antibody" (Final Office Action at page 3). The Examiner then concludes that "[g]iven that antibodies bind epitopic structures rather than sequences per se, and the identity between RCN and calumenin is high, the antibody made by Yabe et al. will also bind polypeptides having SEQ ID NO:3."

First note that Yabe et al. made no antibodies. The four antibodies used in the experiments described by Yabe et al. were all commercially available antibodies made to antigens other than "a polypeptide comprising the amino acid sequence of SEQ ID NO:3." The Examiner's statement that "[o]n page 18234, col. 1, para. 3, Yabe et al. made antibodies against calumenin" does not indicate which of the four commercially available antibodies described in Yabe et al. allegedly anticipates the claimed antibodies. Furthermore, the Examiner has misinterpreted the statements made on page 18234, col. 1, para. 3 of Yabe et al. with regard to the antibodies described in the article. The Examiner purports that this passage is evidence that "Yabe et al. made antibodies against calumenin, anti-protein-disulfide isomerase antibody." What page 18234, col. 1, para. 3 of Yabe et al. actually

says is:

> *Antibodies*--Mouse anti-FLAG M2 antibody (Kodak), ***rabbit anti-protein-disulfide isomerase antibody (Stress Gen Biotechnologies)***, fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse IgG (H + L) (absorbed with human and rabbit serum) (Kirkegaard & Perry Laboratories Inc.), and rhodamine-conjugated affinity-purified goat anti-rabbit IgG (minimal cross-reaction to mouse IgG) (Biosource International) were used. [Emphasis added.]

On page 18235, column 2, paragraph 3 of Yabe et al., the authors describe how a ***commercially available antibody to protein-disulfide isomerase*** was used in conjunction with an anti-FLAG antibody to detect subcellular localization of a FLAG-C39 fusion protein (aka FLAG-calumenin).

> To determine the intracellular localization of the C39 protein, COS-7 cells transfected with FLAG-C39 were doubly stained with an anti-FLAG antibody and an anti-protein-disulfide isomerase antibody and observed under a confocal laser microscope. The staining profile of anti-FLAG antibody (Fig. 4B) was almost identical to that of anti-protein-disulfide isomerase antibody (Fig. 4A), showing diffuse ER staining patterns as reported previously for protein-disulfide isomerase, a well characterized protein in the ER (42). The identical staining patterns with both antibodies (Fig. 4C) indicate that the C39 protein is an ER-resident protein. Since the C39 protein has Ca^{2+} -binding ability and is localized in the ER lumen, we named the C39 protein calumenin. (Yabe et al. at page 18235, column 2, paragraph 3.)

Appellants submit that the antibodies used in these experiments were not directed against the 315 amino acid protein, calumenin, having 98.2% identity with SEQ ID NO:3. In fact, these commercially-available antibodies were directed against Bovine PDI and were purchased from Stress Gen Biotechnologies (see antibody description from Stress Gen Biotechnologies website and "Anti-PDI" product insert; Attachment 7). The immunogen used to make these antibodies is listed on the product insert as Bovine PDI from the sequence reported in M17596 in the GenBank database. M17596 (g163496) is a 510 amino acid protein having 22.8% identity (72/315 aa) to SEQ ID NO:3 (see GenBank records and CLUSTALW alignment; Attachment 8). Clearly, these antibodies do not anticipate the claimed subject matter. Furthermore, ***none*** of the four antibodies described in Yabe et al. anticipate the claimed subject matter.

Moreover, the Examiner fails to appreciate that the language of claim 10 requires that the antibodies ***specifically bind*** to SEQ ID NO:3. It is well understood in the art that an antibody which ***specifically binds*** to a polypeptide sequence binds to that polypeptide sequence ***only*** and does not bind to any other polypeptide sequences. Therefore, the antibodies taught in Yabe et al. which bind to calumenin do not ***specifically bind*** to SEQ ID NO:3 because they bind to other sequences as well. Accordingly, the antibodies recited in claim 10 are not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

II. Yabe et al. does not teach every aspect of the claimed invention either explicitly or impliedly

The Examiner has admitted that Yabe et al. teaches polypeptide sequences which are not 100% identical to the sequences to which the claimed antibodies bind. That is, the sequences taught by Yabe et al. are 98.2% identical to SEQ ID NO:3. (Final Office Action at page 3.) Furthermore, as is set forth above, no antibodies for the calumenin protein described in Yabe et al. are disclosed in the reference.

The Examiner's continued insistence that "with such high identity between calumenin and SEQ ID NO:3, it appears that the epitopic structure of one will be the same as the other and generate the same antibody" and that "the antibody taught in Yabe et al. is the same antibody claimed" (Final Office Action at page 4) is an attempt to determine the scope of the claim while ignoring the language of the claim. This rejection is further based on the allegation that the antibodies taught by Yabe et al. are within the scope of the claimed antibodies. Appellants strongly disagree with the Examiner's position.

Claim 10 recites "An isolated antibody which ***specifically binds*** to a polypeptide comprising the amino acid sequence of SEQ ID NO:3." (Emphasis added.) The claimed antibodies ***specifically bind*** to SEQ ID NO:3 such that those sequences are distinguished from all other sequences. As discussed above in § I., an antibody which ***"specifically binds"*** to SEQ ID NO:3 binds only to a polypeptide comprising SEQ ID NO:3 and will not bind to any other sequence. Appellants note that the claims do not recite an antibody which binds specifically to an epitope of a polypeptide, but rather an antibody which binds **specifically** to a **polypeptide** comprising the amino acid sequence of SEQ ID NO:3. The interaction of the antibody and the recited polypeptide is dependent on the epitope bound

by the antibody, but that does not mean that an antibody that binds specifically to an epitope on the recited polypeptide is the same thing as an antibody that binds specifically to the recited polypeptide. The antibodies recited by the claims bind **specifically** to the recited polypeptides. Since Yabe et al. teach polypeptides other than those recited by the claims; and the antibodies taught by Yabe et al. are raised against the immunogen Bovine PDI, the antibodies taught by Yabe et al. do not bind **specifically** to the recited polypeptides. The antibodies taught by Yabe et al. are excluded from the claimed antibodies because they do not bind **specifically** to the polypeptides recited in the claims.

Furthermore, antibodies which bind specifically to the polypeptide sequences recited in claim 10 can distinguish the differences between the recited polypeptide sequences and those disclosed in Yabe et al., even if the difference is only one amino acid. Evidence in support of this premise may be found in Abaza et al., J. Protein Chem. (1992) 11:433-444 (of record; copy supplied for the convenience of the Board as Attachment 4). As taught by Abaza et al., a single amino acid substitution outside the antigenic site on a protein effects antibody binding. This provides scientific support of Appellants' assertion that so long as there are differences, even just one amino acid residue, between the polypeptide sequences recited in claim 10 and those of the prior art, an antibody which specifically binds to the polypeptide sequences recited in claim 10 cannot be anticipated by the prior art. The Examiner's interpretation of Abaza et al. that "in the absence of sufficient guidance to a particular epitope and the structural context in which the epitope is found; [*sic*] it is highly unpredictable which other isolated polypeptides comprising a variant sequence of SEQ ID NO:2 would maintain the relevant antibody epitope(s)" (Final Office Action at page 5) is confusing. Since no variant sequences of SEQ ID NO:2 are described or claimed in the instant application, these remarks are irrelevant to the claimed subject matter. Appellants reiterate that Abaza et al. provides support for the ***specific binding*** of an antibody to a polypeptide and its ability to distinguish one polypeptide from another on the basis of even one amino acid difference between the polypeptide sequences. Therefore, because there are differences between the polypeptide sequences recited in claim 10 and those of the prior art, an antibody which specifically binds to the polypeptide sequences recited in claim 10 cannot be anticipated by the prior art. Accordingly, claims 10, 30, 31, 33, 36, 37 and 39-42 are not anticipated under 35 U.S.C. § 102(a) by Yabe et al.

III. Bost et al., Bendayan, Li et al. and the Berg patent do not support the Examiner's position for rejection of claims 10, 30, 31, 33, 36, 37 and 39-42 for anticipation under 35 U.S.C. § 102(a).

In a further attempt to support the rejection of claims 10, 30, 31, 33, 36, 37 and 39-42 for anticipation under 35 U.S.C. § 102(a), the Final Office Action cites Bost et al. (Immunol. Invest. 1988; 17:577-586) and Bendayan (J. Histochem. Cytochem. 1995; 43:881-886) as evidence that antibodies that are highly specific can bind to "different molecules not related to the original antigen" (Final Office Action at page 5). Furthermore, the Examiner has cited Li et al. (Proc. Natl. Acad. Sci. USA 77:3211-3214, 1980) in support of the position that one must consider "dissociation of immunoreactive from other biological activities when constructing analogs" (Final Office Action at page 5). The Examiner further relies upon U.S. Pat. No. 6,210,670 (Berg) in support of this rejection; but does not make a specific argument for the use of this reference to support the rejection (Final Office Action at page 5). Appellants submit that none of these articles and/or patents have any relevance to the claimed subject matter.

Bost et al. demonstrate that polyclonal antibodies to the HIV envelope peptide LERILL could also recognize the LEHLLL epitope of IL-2. The Bendayan article describes crossreactivity in immunocytochemical applications of monoclonal antibodies directed to the Arg-Arg region of the human proinsulin molecule. When these two articles are examined in their proper context, neither article supports the Examiner's position that the claimed antibodies which specifically bind to SEQ ID NO:3 would bind to "different molecules not related to the original antigen." Both of these articles are based on studies of antibodies that are directed to very short stretches of amino acid sequences that could be found in many other full length proteins. The Bendayan article, in particular, is based on studies in tissues that were fixed and embedded in paraffin prior to sectioning and immunostaining for electron microscopic immunolabeling. This process changes the morphological state of the proteins present in the tissues which leads to a wider display of crossreactivity as described by the authors:

> The wider crossreactivity displayed by this antibody when used with morphological means could be based on the concept proposed (9) that the antibody recognizes not only a very particular amino acid sequence but also the three-dimensional conformation of this determinant. The processing of the tissues for morphological studies, which is known to affect the structure of proteins, may favor particular conformation(s) of similar

> determinants to resemble the domain present in the parent molecule. (Bendayan at page 886, paragraph 2.)

Appellants therefore submit that neither the Bost et al. nor the Bendayan article supports the Examiner's position that if "an antibody "cross-reacts", [*sic*] i.e., binds to more than one protein sequence, [it] does not mean that the antibody does not "specifically react" with both proteins" (Final Office Action at page 4). Rather, these papers indicate that if antibodies are directed to a small peptide and/or the morphology of the protein to be detected is altered prior to immunoassay, there is a greater likelihood that the antibodies will crossreact with proteins other than the parent molecule if those proteins share either highly homologous or identical short stretches of amino acid residues. This is not germane to what is claimed in the instant application.

Likewise, the Berg patent (U.S. Pat. No. 6,210,670) does not provide support for the Examiner's position regarding the use of the term ***"specifically binds."*** The Final Office Action purports that "Applicant's argument attempts to limit the term "specifically reacts" in a manner inconsistent with the well-known and art-recognized specificity of antibody interaction with epitopes defined by particular amino acid sequences." (Final Office Action at page 5.) The Berg patent teaches the generation of antibodies which bind to short stretches of an amino acid sequence that is common to 3 types of selectins (See the Berg patent at column 3, paragraph 3). These antibodies, as stated in the Summary of the Invention of the Berg patent, were designed to crossreact and bind to all 3 selectins because they only recognize those sequences which are common to all 3 selectins. Here, the Applicants in the Berg patent act as their own lexicographer in defining the term "specifically bind" (See the Berg patent at column 6, paragraph 7). Appellants in the instant application use the term ***"specifically binds"*** in the context of its ordinary meaning throughout the application; and there is no indication to the contrary. Therefore, the meaning of the term "specific binding," as used in the Berg patent, is not applicable to the same term as it is used in the instant application.

The courts have provided that dictionaries, encyclopedias and treatises may be used for interpretation of claim language:

> It has been long recognized in our precedent and in the precedent of our predecessor court, the Court of Customs and Patent Appeals, that dictionaries, encyclopedias and treatises are particularly useful resources to assist the court in determining the ordinary

and customary meanings of claim terms... (internal citations omitted) *Texas Digital Systems Inc. v. Telegenix Inc.*, 64 USPQ2D 1812 (Fed. Cir. 2002).

One must look to the ordinary dictionary definition of the term "specific." In order to put the language of the claims into the proper context, the term "specific" is defined in The American Heritage Dictionary, Second College Edition, as "...2. Pertaining to, ***characterizing or distinguishing a species***. 3. Special, ***distinctive, or unique***, as a quality or attribute. 4. Intended for, applying to, or acting upon a particular thing." (Emphasis added.) When the claims are read in light of this ordinary dictionary definition, it is clear that an "antibody which ***specifically binds*** to a polypeptide" is one which can ***uniquely distinguish*** that polypeptide from other polypeptide sequences. The Berg patent teaches antibodies which bind to short stretches of an amino acid sequence that is common to 3 types of selectins (See the Berg patent at column 3, paragraph 3) and also defines the term "specifically bind" for their own purposes in that particular patent application (See the Berg patent at column 6, paragraph 7). As used in the Berg patent, the definition of the term "specifically bind" cannot be construed as a "well-known and art-recognized specificity of antibody interaction with epitopes defined by particular amino acid sequences." Accordingly, the Berg patent does not provide support for the Examiner's allegation that Appellants' use of the term "specifically binds" is inconsistent with the art-recognized definition of the term (Final Office Action at page 5).

Appellants further submit that the teachings of Li et al. which support separate consideration of immunoreactive and biological activities when constructing analogs are not germane to the claimed subject matter and do not support the rejection on the basis as stated in the Final Office Action.

Once claim 10 (and therefore claims 30, 36 and 39) has been correctly characterized and considered in its proper context, the ancillary issues regarding antibodies in composition (claims 31, 37 and 40), having a label (claim 33), or being produced by either a Fab expression library or an immunoglobulin expression library (claims 41 and 42) become moot. For at least the above reasons, Appellants respectfully request that this rejection be overturned.

Issue 2--Claim rejections under 35 U.S.C. § 102(b)

Claims 10, 30, 31, 33, 36, 37 and 39-42 have been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Ozawa et al. (1993; J. Biol. Chem. 268:699-705; of record; copy supplied for the convenience of the Board as Attachment 9). The Final Office Action asserts that:

> Ozawa et al. teach reticulocalbin having 89.1% identity to SEQ ID NO:3. (Final Office Action at page 5.)
>
> Ozawa et al. made antibodies against reticulocalbin. Given that antibodies bind epitopic structures rather than sequences per se, and the identity between RCNδ and reticulocalbin is high, the antibody made by Ozawa et al. will also bind polypeptides having SEQ ID NO:3... (Final Office Action at page 4.)
>
> the antibodies were in composition (Claim 31, 37, 40)...the antibodies were labeled via conjugation with fluorescein isothiocyanate (Claim 33). Claims 41 and 42 are being considered to be anticipated as well because there appears to be no difference in the antibody made by a Fab expression library or and [sic] immunoglobulin expression library. (Final Office Action at page 4.)

Appellants strongly disagree with the Examiner's position for the following reasons.

First note that although the Response mailed on June 11, 2003 and received in the USPTO on June 13, 2003 contained evidence that the sequence the Examiner used to produce the alignment that was sent with the Office Action mailed March 11, 2003 is not the reticulocalbin sequence that was published on page 701 of the Ozawa et al. article, the Examiner did not acknowledge this in the Final Office Action mailed August 6, 2003. A copy of this evidence, which was submitted to the USPTO on June 11, 2003, is supplied for the convenience of the Board as Attachment 10. A careful visual comparison of the sequences reveals that the sequence used to produce the alignment labeled "RESULT 2" on page 2 of the document generated on Tue Feb 25 16:40:19 2003 (copy supplied for the convenience of the Board as Attachment 11) is 315 AA in length and begins with "MDLRQFLMC..." The reticulocalbin sequence published at page 701 of the Ozawa et al. article is 325 AA in length and begins with "MARGGRLGLALG..." Clearly, these are not the same sequences. Of particular note is that the 315 AA sequence used by the Examiner in the alignment labeled "RESULT 2" (Attachment 11) was submitted in ***October 1997*** and created in the TrEMBLrel. Database on January 1, 1998. The priority date of the instant application is ***August 8, 1997***.

Therefore, this sequence is not prior art to SEQ ID NO:3.

Appellants resubmit the attached CLUSTALW alignment (Attachment 10) of SEQ ID NO:3 (1601793CD1) with the reticulocalbin sequence published in Ozawa et al. (g220582). The attached alignment indicates that there is a 60% sequence identity (192/315 amino acids) between the two sequences--and the sequence identity is only that high because the CLUSTALW program inserted five gaps in SEQ ID NO:3 to effect the alignment. Additionally, Appellants submit that the rejection cannot withstand further scrutiny of the law under 35 U.S.C. § 102.

The law is clear with regard to the requirements for making a rejection under any subsection of 35 U.S.C. § 102:

> For a prior art reference to anticipate in terms of 35 U.S.C. § 102, every element of the claimed invention must be identically shown in a single reference." *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990).

Furthermore, the MPEP provides guidance for interpretation of the case law that has been generated with regard to 35 U.S.C. § 102:

> for anticipation under 35 U.S.C. 102, ***the reference must teach every aspect of the claimed invention either explicitly or impliedly***. Any feature not directly taught must be inherently present. (*MPEP 706.02* at page 700-21, Rev. 1, Feb. 2003) [Emphasis added.]

The polypeptide sequence taught by Ozawa et al. does not ***teach every aspect of the claimed invention either explicitly or impliedly***. The Examiner has alleged that the polypeptide sequence taught by Ozawa et al. (reticulocalbin) is 89.1% identical to SEQ ID NO:3 (RCNδ). (Final Office Action at page 4.) As noted above, the reticulocalbin taught by Ozawa et al. is only 60% identical to SEQ ID NO:3. It is, therefore, possible to make an antibody to SEQ ID NO:3 that does not bind the reticulocalbin taught in Ozawa et al. Such an antibody is recited in claim 10 as "An isolated antibody ***which specifically binds*** to a polypeptide comprising the amino acid sequence of SEQ ID NO:3." By **"specifically binding"** to SEQ ID NO:3, the claimed antibody must bind ***only*** to a polypeptide consisting of SEQ ID NO:3. Accordingly, so long as there are differences, even just one amino acid residue, between the amino acid sequence recited in claim 10 and that disclosed in Ozawa et al., an antibody can be produced that can specifically bind to the polypeptide recited in claim 10 and not that

of the reticulocalbin taught in Ozawa et al.

Evidence in support of this premise may be found in Abaza et al., J. Protein Chem. (1992) 11:433-444 (of record; Attachment 4). As taught by Abaza et al., a single amino acid substitution outside the antigenic site on a protein effects antibody binding. This provides scientific support of Appellants' assertion that so long as there are differences, even just one amino acid residue, between the amino acid sequence of claim 10 and those of the prior art, an antibody can be produced that can specifically bind to the polypeptide recited in claim 10 and not those of the prior art. Accordingly, given the extensive amino acid differences between SEQ ID NO:3 and reticulocalbin (at least 40%), one of skill in the art could produce an antibody which binds to the polypeptide recited in claim 10 alone and without cross-reactivity to other polypeptides even those which have extensive sequence identity to SEQ ID NO:3, which reticulocalbin does not have.

Ozawa et al. explicitly states that "[m]onospecific antibodies against reticulocalbin were prepared..." by immunizing rabbits with gel purified reticulocalbin fusion protein prepared in the maltose-binding protein (MBP) fusion vector, pMAL-c (Ozawa et al., page 700, column 1 paragraph 2 and column 2, paragraph 3). The nucleotide sequence used in the expression vector and the amino acid sequence of the fusion protein thus expressed are shown on page 701 at Figure 2 of Ozawa et al. The CLUSTALW alignment of SEQ ID NO:3 with the amino acid sequence of the fusion protein shown on page 701 at Figure 2 (Attachment 10) clearly shows that SEQ ID NO:3 and the amino acid sequence reported in Ozawa et al. are clearly not the same protein.

The Examiner's continued insistence that "[g]iven that antibodies bind epitopic structures rather than sequences per se, and the identity between RCN[δ] and reticulocalbin is high, the antibody made by Ozawa et al. will also bind polypeptides having SEQ ID NO:3, 90% identity to SEQ ID NO:3, biologically active fragments of SEQ ID NO:3, and immunogenic fragments of SEQ ID NO:3..." and that "the antibody taught in Ozawa et al. is the same antibody claimed" (Final Office Action at page 6) is an attempt to determine the scope of the claim while ignoring the language of the claim. This rejection is further based on the allegation that the antibodies taught by Ozawa et al. are within the scope of the claimed antibodies. Appellants strongly disagree with the Examiner's position.

First note that independent claim 10 does not recite "...90% identity to SEQ ID NO:3, biologically active fragments of SEQ ID NO:3, and immunogenic fragments of SEQ ID NO:3..." as

purported by the Final Office Action at page 6. Claim 10 recites "An isolated antibody **which specifically binds to a polypeptide** comprising the amino acid sequence of SEQ ID NO:3." (Emphasis added.) Appellants note that the claims do not recite an antibody which binds specifically to an epitope of a polypeptide, but rather an antibody which binds **specifically** to a **polypeptide** comprising the amino acid sequence of SEQ ID NO:3. The interaction of the antibody and the recited polypeptide is dependent on the epitope bound by the antibody, but that does not mean that an antibody that binds specifically to an epitope on the recited polypeptide is the same thing as an antibody that binds specifically to the recited polypeptide.

The courts have provided that dictionaries, encyclopedias and treatises may be used for interpretation of claim language:

> It has been long recognized in our precedent and in the precedent of our predecessor court, the Court of Customs and Patent Appeals, that dictionaries, encyclopedias and treatises are particularly useful resources to assist the court in determining the ordinary and customary meanings of claim terms... (internal citations omitted) *Texas Digital Systems Inc. v. Telegenix Inc.*, 64 USPQ2D 1812 (Fed. Cir. 2002).

One must look to the ordinary dictionary definition of the term "specific." In order to put the language of the claims into the proper context, the term "specific" is defined in The American Heritage Dictionary, Second College Edition, as "...2. Pertaining to, ***characterizing or distinguishing a species***. 3. Special, ***distinctive, or unique***, as a quality or attribute. 4. Intended for, applying to, or acting upon a particular thing." (Emphasis added.) When the claims are read in light of this ordinary dictionary definition, it is clear that an "antibody which ***specifically binds*** to a polypeptide" is one which can ***uniquely distinguish*** that polypeptide from other polypeptide sequences.

The antibodies recited by the claims bind **specifically** to the recited polypeptides. Since Ozawa et al. teach polypeptides other than those recited by the claims; and the antibodies taught by Ozawa et al. are raised against a gel purified reticulocalbin fusion protein prepared in the maltose-binding protein (MBP) fusion vector, pMAL-c (as shown in the amino acid sequence reported in Figure 2 of Ozawa et al.), the antibodies taught by Ozawa et al. do not bind **specifically** to the recited polypeptides. The antibodies taught by Ozawa et al. are excluded from the claimed antibodies because they do not bind **specifically** to the polypeptides recited in the claims.

Once claim 10 (and therefore claims 30, 36 and 39) has been correctly characterized and considered in its proper context, the ancillary issues regarding antibodies in composition (claims 31, 37 and 40), having a label (claim 33), or being produced by either a Fab expression library or an immunoglobulin expression library (claims 41 and 42) become moot. For at least the above reasons, Appellants respectfully request that this rejection be reversed.

Issue 3--Claim rejections under 35 U.S.C. § 112, second paragraph

Claims 36 and 39 have been rejected under 35 U.S.C. § 112, second paragraph for alleged indefiniteness as depending from claims directed to non-elected inventions. Appellants submit that, once product claim 10 is allowed, the method claims 35 and 38 from which claims 36 and 39 depend will be rejoined. Since the patentability of method claims 35 and 38 relies on the novelty and patentability of claim 10, and these claims do not expand the scope of the products recited in claim 10, claims 35 and 38 will be allowable upon rejoinder. Once this has been accomplished, claims 36 and 39 will depend from allowable method claims 35 and 38. Accordingly since Appellants are confident that the Board will overturn the 35 U.S.C. § 112, first paragraph and 35 U.S.C. § 102(a) and § 102(b) rejections against claim 10 and allow claim 10 after considering the arguments submitted above, Appellants respectfully submit that this rejection of claims 36 and 39 will be moot.

(9) CONCLUSION

Due to the urgency of this matter and its economic and public health implications, an expedited review of this appeal is earnestly solicited.

If the USPTO determines that any additional fees are due, the Commissioner is hereby authorized to charge Deposit Account No. **09-0108.**

This brief is enclosed in triplicate.

Respectfully submitted,

INCYTE CORPORATION

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Attachments:

1. Yabe et al.; July 18, 1997; J. Biol. Chem. 272:18323-18239
2. Bost et al.; Immunol. Invest. (1988); 17:577-586
3. Bendayan ; J. Histochem. Cytochem. (1995); 43:881-886
4. Abaza et al., J. Protein Chem. (1992) 11:433-444
5. Li et al.; Proc. Natl. Acad. Sci. (1980) USA 77:3211-3214
6. U.S. Pat. No. 6,210,670 (Berg)
7. Stress Gen Biotechnologies website and "Anti-PDI" product insert
8. g163496 GenBank records and CLUSTALW alignment with SEQ ID NO:3
9. Ozawa et al.; J. Biol. Chem. (1993); 268:699-705
10. CLUSTALW alignment of g220581 and SEQ ID NO:3, including NCBI documents for g220581 (3 pages)
11. alignment labeled "RESULT 2" on page 2 of the document generated on Tue Feb 25 16:40:19 2003

APPENDIX - CLAIMS ON APPEAL

10. An isolated antibody which specifically binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:3.

30. The antibody of claim 10, wherein the antibody is:

a) a chimeric antibody,

b) a single chain antibody,

c) a Fab fragment,

d) a $F(ab')_2$ fragment, or

e) a humanized antibody.

31. A composition comprising an antibody of claim 10 and an acceptable excipient.

33. A composition of claim 31, wherein the antibody is labeled.

36. An antibody produced by a method of claim 35.

37. A composition comprising the antibody of claim 36 and a suitable carrier.

39. A monoclonal antibody produced by a method of claim 38.

40. A composition comprising the antibody of claim 39 and a suitable carrier.

41. The antibody of claim 10, wherein the antibody is produced by screening a Fab expression library.

42. The antibody of claim 10, wherein the antibody is produced by screening a recombinant immunoglobulin library.

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Calumenin, a Ca^{2+}-binding Protein Retained in the Endoplasmic Reticulum with a Novel Carboxyl-terminal Sequence, HDEF*

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We have identified and characterized a cDNA encoding a novel Ca^{2+}-binding protein named calumenin from mouse heart by the signal sequence trap method. The deduced amino acid sequence (315 residues) of calumenin contains an amino-terminal signal sequence and six Ca^{2+}-binding (EF-hand) motifs and shows homology with reticulocalbin, Erc-55, and Cab45. These proteins seem to form a new subset of the EF-hand protein family expressed in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus. Purified calumenin had Ca^{2+}-binding ability. The carboxyl-terminal tetrapeptide His-Asp-Glu-Phe was shown to be responsible for retention of calumenin in ER by the retention assay, immunostaining with a confocal laser microscope, and the deglycosylation assay. This is the first report indicating that the Phe residue is included in the ER retention signal. Calumenin is expressed most strongly in heart of adult and 18.5-day embryos. The calumenin gene (*Calu*) was mapped at the proximal portion of mouse chromosome 7.

The endoplasmic reticulum (ER)[1] is involved in synthesis and modification of secretory and membranous proteins as well as resident proteins in the lumen of the ER, Golgi apparatus, or lysosomes (1). The ER is also known as the major Ca^{2+} storage compartment in eukaryotic cells. By pumping cytosolic Ca^{2+} into the ER lumen, cells keep their cytosolic concentration of free Ca^{2+} at extremely low levels so that they can use Ca^{2+} as intracellular signal (2). Besides, the ER itself needs luminal Ca^{2+} for its normal functions such as protein folding and protein sorting (3–6). Among many ER-resident proteins, endoplasmin (GRP94) (7, 8), Bip (GRP78) (9, 10), protein-disulfide isomerase (ERp59) (11, 12), and calreticulin (CRP55) (13) are Ca^{2+}-binding proteins that are associated with Ca^{2+}-dependent folding and maturation of secretory proteins in the ER lumen (14, 15). In addition, two Ca^{2+}-binding ER-resident proteins, reticulocalbin (16) and Erc-55 (17), have been isolated recently. They have multiple EF-hand motifs and constitute a new subset of the EF-hand superfamily, together with a homologous protein in the lumen of the Golgi apparatus, Cab45 (18). However, their physiological functions are still unknown.

ER-resident proteins generally carry a retention signal at their carboxyl terminus. This ER retention signal is first identified to be tetrapeptides, Lys-Asp-Glu-Leu (KDEL) in mammalian cells and His-Asp-Glu-Leu (HDEL) in yeast (19, 20). Soluble ER-resident proteins are trapped by binding to the KDEL receptor expressed in the cis-Golgi and retrieved to the ER (21–23). Further studies have demonstrated that HDEL and several variants of the KDEL sequence can also work as the ER retention signal in mammalian cells (24, 25). Comparison of variants of the KDEL sequence suggests that the replacement of Lys and Asp residues with other amino acid residues does not abolish the ER retention activity. However, the carboxyl-terminal two residues are considered to be critical because the third and fourth positions in all of the ER retention signals are Glu/Asp and Leu/Ile, respectively.

During the embryogenesis, the heart begins to beat already in the 8.5-day mouse embryo, whose heart is still a two-chambered tube with one atrium and one ventricle (26). Beating of cardiac myocytes is maintained by the strict regulation of their cytoplasmic Ca^{2+} concentration. To achieve this regulation, cardiac myocytes develop their specialized ER, called the sarcoplasmic reticulum, as the Ca^{2+} storage compartment and produce the rhythmical Ca^{2+} oscillation between the sarcoplasmic reticulum and the cytosol (27). Molecules involved in this Ca^{2+} oscillation are reported to be present very early in mouse cardiogenesis (28, 29).

Since we are interested in molecules involved in heart embryogenesis, we screened in a signal sequence trap library (30, 31) of mouse embryonic heart to isolate cDNAs encoding amino-terminal hydrophobic signal sequences. We here report cloning and characterization of cDNA encoding calumenin that binds Ca^{2+} and carries a new ER retention signal, HDEF, at the carboxyl terminus. Calumenin is a novel member of the reticulocalbin family, a new subset of the EF-hand superfamily in the ER. Calumenin is most strongly expressed in the heart of adult and 18.5-day embryos.

EXPERIMENTAL PROCEDURES

cDNA Cloning—Poly(A) RNA from approximately 100 hearts of 9.5-day postcoitus (dpc) mouse embryos was extracted with TRIzol reagent (Life Technologies, Inc.) and Oligotex™-dT30<Super> (Roche). cDNA was synthesized from 1.35 μg of poly(A) RNA using Super Script II (Life Technologies, Inc.). First strand cDNA was synthesized with 25 pmol of URPX3 primer: GAG-ACG-GTA-ATA-CGA-TCG-ACA-GTA-GCT-CGA-G*XXX-XXX-XXX-X* (where *X* represents one of the following: A, G, C, or T). After alkali lysis of RNA and the poly(A) tailing procedure, second strand cDNA was synthesized with 25 pmol of ESTN primer: CCG-CGA-ATT-CTG-ACT-AAC-TGA-$(T)_{17}$*XX*. Then cDNA of 400–800 base pairs were fractionated by agarose gel electrophoresis and subjected to polymerase chain reaction (PCR) using ExTaq (TaKaRa, Japan) and 25 pmol of ESP primer (CCG-CGA-ATT-CTG-ACT-AAC-TGA-TT) and Ad-P1 primer (GAC-GGT-AAT-ACG-ATC-GAC-AGT-AGG) under the following conditions for Thermal-Cycler (TaKaRa, Japan): 94 °C for 5 min and then 94 °C for 45 s, 52 °C for 60 s, and 72 °C for 2 min for 25 cycles and 72 °C for 10 min. After cloning cDNA unidirectionally between the *Eco*RI and *Xho*I sites of pSuc2t7F1ori vector, screening procedures were performed as described (31).

To clone full-length cDNA, the 3′-rapid amplification of cDNA ends

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U81829.

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[1] The abbreviations used are: ER, endoplasmic reticulum; Bip, immunogloblin heavy chain binding protein; dpc, day(s) postcoitus; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

A

```
GATCCTGCGGCGTGGAGCTCCCGGGAAAGGTTATCATGGACCTGCGTCAGTTTCTTATGT :60
                                        M  D  L  R  Q  F  L  M  C :9
GCCTGTCCCTGTGCACAGCCTTTGCTTTGAGCAAGCCTACAGAAAAGAAGGACCGAGTAC :120
  L  S  L  C  T  A  F  A  L  S  K  P  T  E  K  K  D  R  V  H :29
ACCATGAGCCTCAGCTCAGCGATAAAGTTCACAATGATGCTCAGAATTTTGACTATGACC :180
  H  E  P  Q  L  S  D  K  V  H  N  D  A  Q  N  F  D  Y  D  H :49
ATGATGCCTTCTTGGGTGCAGAAGAGGCAAAGAGTTTTGATCAGCTGACACCAGAAGAGA :240
  D  A  F  L  G  A  E  E  A  K  S  F  D  Q  L  T  P  E  E  S :69
GCAAGGAAAGGCTTGGAAAGATTGTAAGTAAAATAGATGACGACAAGGATGGGTTTGTCA :300
  K  E  R  L  G  K  I  V  S  K  I  D  D  D  K  D  G  F  V  T :89
CTGTGGATGAACTCAAAGGCTGGATTAAGTTTGCACAAAAGCGCTGGATTCACGAGGATG :360
  V  D  E  L  K  G  W  I  K  F  A  Q  K  R  W  I  H  E  D  V :109
TAGAGCGGCAATGGAAGGGGCACGACCTCAATGAGGATGGCCTCGTTTCCTGGGAGGAGT :420
  E  R  Q  W  K  G  H  D  L  N  E  D  G  L  V  S  W  E  E  Y :129
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  A  R  H  L  V  Y  E  S  D  Q  N  K  D  G  K  L  T  K  E  E :289
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TAGTACGACATGATGAGTTCTGAGCTGCAGACAGAGGAACCTACATTTCTTCAAAAGTAA :1020
  V  R  H  D  E  F  *                                        :315
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TCTGAGGGGCTGGAGGGAACCCGAGCTTCCGAGGAACAACTCTGATTAGTACACTTGTGT :1200
CTGTAGGTTTACACTTTGTATAATGTATAACATGGTGTGTTTATTTTTGTATTGTTCTCT :1260
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GCTAAATAGCAGTATTCATTACAAGCTTGCTTGCAGTGTTCTTACCTTGTGGGCTGGTTC :2100
CTAAACCTCACCCTGCTGATTGGACCAGGGATCCTCATACCTCAGAAGGCAAACCACTCT :2160
TCTCCCAGGCCTCTGCTCACCAGGGCTTGAGCTTTGCCTCAAGGATAGAAGGATGTACAG :2220
AGGGGGCTCTTGGTTTGAGGACCACTTGAGCCGTTTTCTCATCTCCCTTTCTCTCTGACA :2280
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ATCATCATCATCATCTGTTGCCAACTCCATTCACCTGTTTTGGTCTCGAGTTGATGGGCC :2400
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B

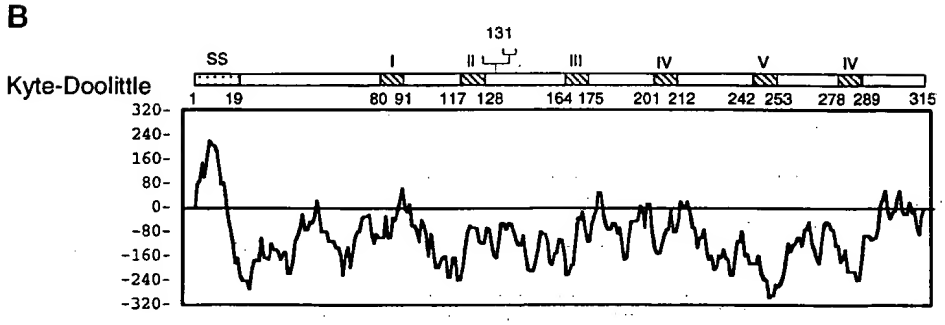


FIG. 1. **Nucleotide and predicted amino acid sequences of C39 cDNA.** *A*, positions of nucleotides (*upper row*) and amino acids (*lower row*) are shown at the *right*. cDNA sequence data is available from GenBank℠/EMBL/DDBJ under accession number U81829. The polyadenylation signal is *boxed*. *B*, schematic view and hydropathy plot of C39 protein. C39 protein has an amino-terminal signal sequence (*SS*), a predicted *N*-glycosylation site at position 131, and six EF-hand motifs. *Arabic numbers under* the *schema* indicate positions of amino acids at these landmarks. *Roman numbers* refer to EF-hand motifs. The hydropathy plot calculated by the algorithm of Kyte and Doolittle shows that the C39 protein has a typical amino-terminal signal sequence but no membrane anchor sequence.

(RACE) method was performed with LAtaq (TaKaRa). Mouse 13.5-dpc embryonic heart cDNA library in Uni-ZAP XR vector was used (Stratagene). C39N1b outer primer (biotinylated) (GTG-GAG-CTC-CCG-GGA-AAG-GTT-ATC-ATG) and C39N2 inner primer (CAT-GGA-CCT-GCG-TCA-GTT-TC) were designed as gene-specific primers and used with standard T7 primer (CGC-GTA-ATA-CGA-CTC-ACT-ATA-GGG-C). Conditions adopted for Perkin-Elmer 9600 were as follows: 95 °C for 2 min and then 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min for 25 cycles. The reaction mixture of first PCR with C39N1b and T7 primers was subjected to purification with M280 Dynabeads (Dynal) to decrease backgrounds due to nonspecific amplification by T7 primer. Second PCR was performed with C39N2 and T7 primers using the purified mixture.

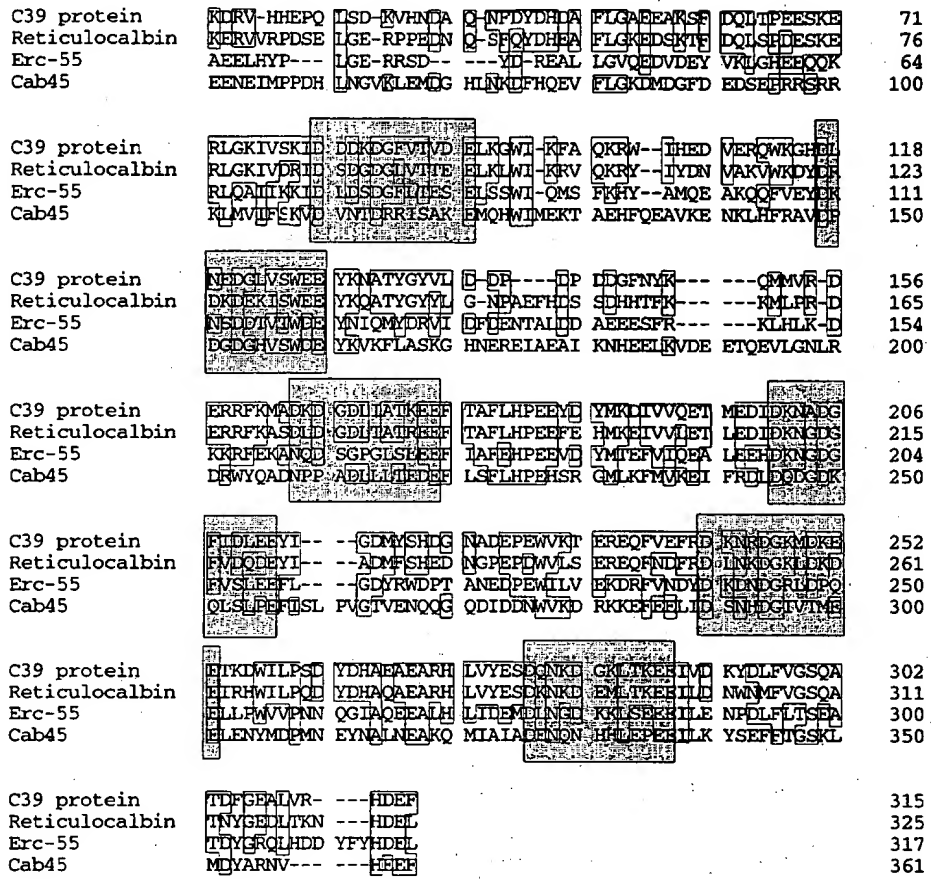


FIG. 2. **Alignment of C39, Erc-55, reticulocalbin, and Cab45.** All family members share significant homology even outside the EF-hand motif. EF-hand motifs are *shaded*. Amino acid residues identical to the C39 protein are *boxed*.

For sequencing, amplified cDNA was cloned into pGEM-T vector (Promega).

Sequencing was performed with an automated sequencer (model 373A; Applied Biosystems), and sequence analysis was done with the computer analysis program, GeneWorks (IntelliGenetics, Inc.). Homology search was performed with BLAST and FASTA, using GenBank® and EMBL as DNA data bases and PRF, PIR, and SwissProt as protein data bases. Motif search and localization analysis were performed on line at Prosite.

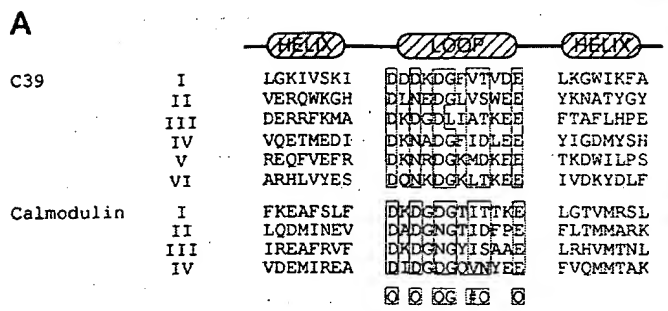
Expression and Purification of Proteins—To express proteins in mammalian cells, cDNAs were cloned in *Xba*I site of pEF-BOS expression vector (32), and their sequences were confirmed before assays. cDNAs of FLAG-calumenin (FLAG-C39), FLAG-calumenin-ΔHDEF, and FLAG-calumenin-rHDEL were constructed with PCR. FLAG epitope (8 amino acids; DYKDDDDK) was incorporated 5 amino acids downstream of the putative signal sequence cleavage site. The following primers were used for PCR: FLAG-calumenin primer, AAG-CCT-ACT--AGT-ATG-GAC-CTG-CGT-CAG-TTT-CTT-ATG-TGC-CTG-TCC-CTG--TGC-ACA-GCC-TTT-GCT-TTG-AGC-AAG-CCT-ACA-GAA-GAC-TAC--AAG-GAC-GAC-GAT-GAC-AAG-AAG-AAG-GAC-CGA-GTA-CAC-CA-T-GAG-C; calumenin-HDEF primer, AAG-CCT-ACT-AGT-TCA-GAA--CTC-ATC-ATG-TCG-TAC-TAA-GG; calumenin-ΔHDEF primer, AAG--CCT-ACT-AGT-TCA-TCG-TAC-TAA-GGC-CTC-CCC; and calumenin-rHDEL primer, AAG-CCT-ACT-AGT-TCA-CAA-CTC-ATC-ATG-TCG--TAC-TAA-GG. Proteins were purified from transfected cells according to the standard protocol for immunoprecipitation, using lysis buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris-HCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) and the anti-FLAG affinity gel (Eastman Kodak Co.).

Antibodies—Mouse anti-FLAG M2 antibody (Kodak), rabbit anti-protein-disulfide isomerase antibody (Stress Gen Biotechnologies), fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse IgG (H + L) (absorbed with human and rabbit serum) (Kirkegaard & Perry Laboratories Inc.), and rhodamine-conjugated affinity-purified goat anti-rabbit IgG (minimal cross-reaction to mouse IgG) (Biosource International) were used.

Other Methods—The procedures used to stain cells were essentially as described (18, 33). Stained cells were analyzed with a confocal scanning laser microscope (LSM410 UV, Carl Zeiss Inc.) following the protocol for double stain by fluorescein isothiocyanate and rhodamine. The Slow Fade Antifade kit (Molecular Probes, Inc.) was used to prevent photobleaching. Retention of calumenin in ER was assayed as follows. Cells transfected with FLAG-calumenin and FLAG-calumenin-ΔHDEF were further incubated for 4 h in serum-free medium. The media were then concentrated using Centricon-30 (Amicon). Cell extracts and concentrated media were analyzed by 12% polyacrylamide gel electrophoresis with SDS followed by Western blotting. The reverse transcriptase-PCR method. After first strand synthesis using random 9-mer, PCR was performed with C39N3 primer (GGA-AGA-TGG-ACA-AGG-AAG-AGA-CC) and C39C1b primer (biotinylated) (AGA-GTT-GTT-CCT-CGG-AAG-CTC-G). Transfection of COS-7 cells was performed using lipofectamine (Life Technologies) according to the manufacturer's protocol. Protein synthesis was stopped before protein purification and immunostaining assay by treating cells with 300 μM cycloheximide for 2 h (34). The filter for Northern blot was prepared as described (35) and hybridized using Quick-Hyb solution (Stratagene). The $^{45}Ca^{2+}$ binding assay was performed as described (36). Membranes of $^{45}Ca^{2+}$ binding assay and Northern blotting was analyzed using an image analyzer (BAS 2000, Fuji Film). Deglycosylation assay was done as described (18) and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. For Western blotting, ECL Western blotting detection reagents (Amersham Life Science, Inc.) was used. Chromosomal mapping of the calumenin gene was done as described (35, 37).

RESULTS

Cloning and Sequencing of a Novel cDNA C39—4.4×10^5 yeast transformants from 9.5 dpc embryonic heart cDNA library were screened, and 386 positive clones were obtained by the signal sequence trap method described previously (30, 31). Among these, nucleotide sequences of 17 clones were not reported in any mammalian species, 15 clones were homologous to sequences reported in mouse or other mammals and the rest were redundant clones. One of the novel clones (C39) was picked up for further studies because its mRNA was most strongly expressed in heart (see below). The full-length cDNA of C39 was isolated from a 13.5 dpc embryonic heart cDNA library by the 3′-RACE method. Nucleotide sequences of two independent clones were determined to avoid sequence errors



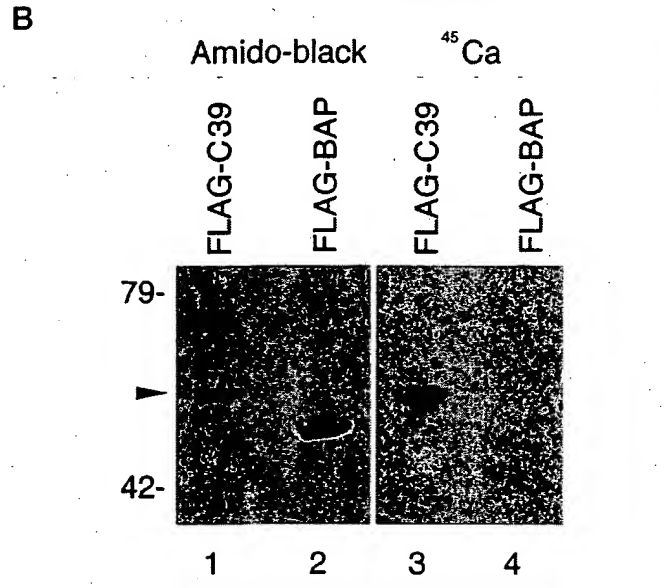


FIG. 3. **C39 encodes a Ca^{2+}-binding protein.** *A*, EF-hand motifs of the C39 protein are compared with those of calmodulin. The EF-hand motif is defined as the sequence consisting of the loop domain and two flanking helices. Consensus amino acid residues in the loop domain are *boxed*. # and O indicate a consensus hydrophobic amino acid and amino acid with the oxygen-containing side chain, respectively. Glycine (*G*) in the loop domain is often but not necessarily conserved in active EF-hand motifs. *B*, a $^{45}Ca^{2+}$ binding assay was performed as described (36). The purified FLAG-C39 fusion protein (*lanes 1* and *3*) and the FLAG-bacterial alkali phosphatase (*FLAG-BAP*) fusion protein (*lanes 2* and *4*) were electrophoresed in 12% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and incubated with $^{45}Ca^{2+}$. After washing, the filter was exposed in an image analyzer (^{45}Ca; *lanes 3* and *4*). The filter was also stained with Amido Black after the exposure (*Amido Black*; *lanes 1* and *2*). The *arrowhead* indicates the FLAG-C39 fusion protein.

and shown to encode a 315-amino acid protein (Fig. 1*A*). The translation start site was assigned at nucleotide positions 36–38 because of the presence of an NH_2-terminal signal sequence and the comparison with other family members to be described below.

The C39 Protein Is a Ca^{2+}-binding Protein—cDNA sequence showed that the C39 protein does not have any other hydrophobic stretch long enough to anchor the protein in the membrane (Fig. 1*B*). The C39 protein carries six potential Ca^{2+}-binding EF-hand motifs and a putative *N*-glycosylation site. Homology search with the BLAST and FASTA computer program revealed the presence of many Ca^{2+}-binding proteins homologous to the C39 protein in the EF-hand region. Among these, reticulocalbin (16) and Erc-55 (17) in the ER and Cab45 (18) in the Golgi apparatus had homology in size and sequence to the C39 protein even outside of the EF-hand region (Fig. 2). These proteins appear to form one subfamily of Ca^{2+}-binding proteins.

Each EF hand in the C39 protein has the general feature for high affinity Ca^{2+}-binding according to Kretsinger's rule (38);

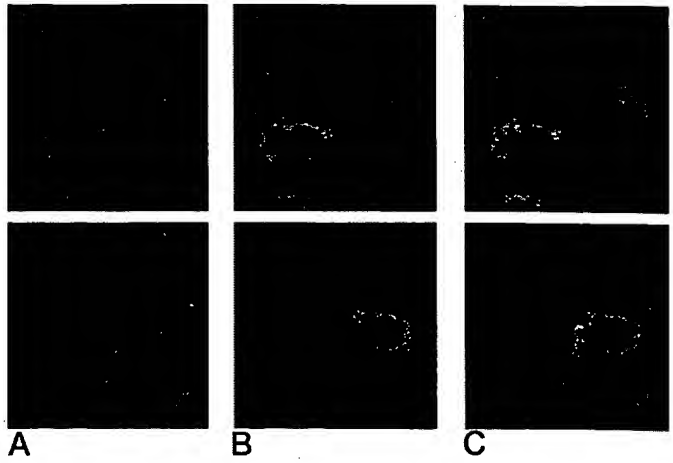


FIG. 4. **Localization of C39 protein by immunostaining.** COS-7 cells, which were transfected with pEF-BOS-FLAG-C39, were doubly stained with anti-FLAG antibody (*B*; visualized with a secondary fluorescein isothiocyanate-conjugated antibody) and anti-protein-disulfide isomerase antibody (*A*; visualized with a secondary rhodamine-conjugated antibody) after 2 h of treatment with 300 μM cycloheximide. Staining profiles were observed using a confocal scanning laser microscope. *C* is an overlap image of *A* and *B*.

the presence of the helix-loop-helix motif in which 5 oxygen-containing residues and the central glycine are conserved for coordination of Ca^{2+} binding (Fig. 3*A*). Although the central glycine of the third motif is replaced by leucine, the same replacement was seen in other Ca^{2+}-binding proteins (39, 40) and considered to retain the Ca^{2+}-binding activity by the secondary structure prediction of computer analysis (41).

To test whether six EF hands in the C39 protein really have Ca^{2+}-binding ability, the $^{45}Ca^{2+}$ binding assay was performed as described (36). Strong signal was detected in the FLAG-C39 fusion protein but none in the FLAG-bacterial alkali phosphatase fusion protein (Fig. 3*B*). The positive band was approximately 57 kDa in size, which was equal to the size of the FLAG-C39 fusion protein detected by Amido Black staining, indicating that EF hands in the C39 protein indeed have Ca^{2+} binding ability.

Subcellular Localization of the C39 Protein—To determine the intracellular localization of the C39 protein, COS-7 cells transfected with FLAG-C39 were doubly stained with an anti-FLAG antibody and an anti-protein-disulfide isomerase antibody and observed under a confocal laser microscope. The staining profile of anti-FLAG antibody (Fig. 4*B*) was almost identical to that of anti-protein-disulfide isomerase antibody (Fig. 4*A*), showing diffuse ER staining patterns as reported previously for protein-disulfide isomerase, a well characterized protein in the ER (42). The identical staining patterns with both antibodies (Fig. 4*C*) indicate that the C39 protein is an ER-resident protein. Since the C39 protein has Ca^{2+}-binding ability and is localized in the ER lumen, we named the C39 protein calumenin.

The HDEF Sequence Is a Novel ER Retention Signal—To examine whether the C-terminal tetrapeptide HDEF of calumenin can serve as a novel intracellular retention signal, we constructed two expression vectors: FLAG-calumenin, a fusion protein of FLAG epitope and calumenin, and FLAG-calumenin-ΔHDEF, a fusion protein of the FLAG epitope and calumenin lacking its C-terminal HDEF sequence. FLAG epitopes were incorporated 5 amino acids downstream of the putative signal sequence cleavage site in each construct. COS-7 cells were transfected by these constructs, and concentrated media and cell extracts were analyzed by Western blotting. Most of the FLAG-calumenin-ΔHDEF protein was secreted into the medium, while almost all of the FLAG-calumenin protein was

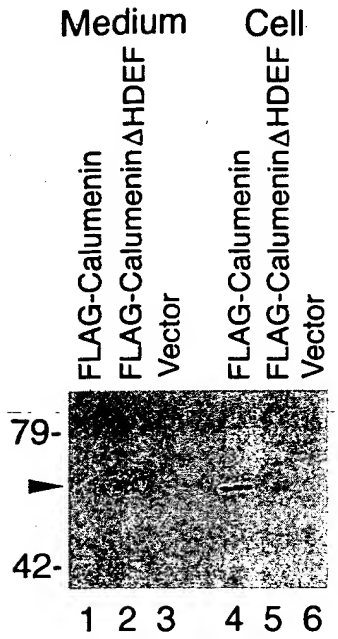


FIG. 5. **HDEF works as a intracellular retention signal.** A retention assay was performed using COS-7 cells transfected with pEF-BOS-FLAG-calumenin, pEF-BOS-FLAG-calumenin-ΔHDEF and pEF-BOS (control). After transfection, cells were washed and chased for a further 4 h in serum-free media. Then media were concentrated and analyzed by Western blotting together with cell extracts. FLAG-calumenin-ΔHDEF was secreted (*lane 2*), while almost all of the FLAG-calumenin was retained in cells (*lane 1*). Different amounts of proteins in cells may be due to the secretion of FLAG-calumenin-ΔHDEF (*lanes 4* and *5*). The *arrowhead* indicates the FLAG-calumenin fusion protein.

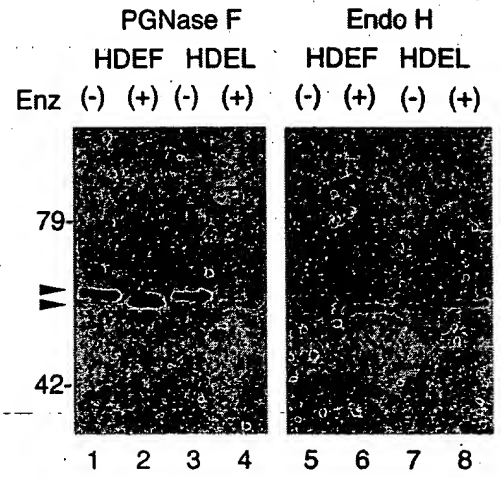


FIG. 6. **Calumenin is a glycosylated protein in the ER.** Transfected cells were treated with 300 μM cycloheximide for 2 h to stop further protein synthesis. The FLAG-calumenin (HDEF) and FLAG-calumenin-rHDEL (HDEL) proteins were purified and subjected to endoglycosidase H and *N*-glycosidase F digestion for 1 h at 37 °C. The *upper band* indicates glycosylated proteins, and the *lower band* indicates deglycosylated forms. *Enz*, enzyme.

retained in the cell (Fig. 5). These results indicate that HDEF is essential to maintain calumenin within the cell.

We further examined intracellular localization of calumenin by treatment with deglycosidases. Proteins translocated into the ER are exposed to core glycosylation. At this moment, glycosylated proteins are sensitive to the deglycosidase endoglycosidase H, which cleaves high mannose oligosaccharides on proteins. However, once proteins are transported into the medial Golgi and modified by Golgi-mannosidase II, they become resistant to endoglycosidase H. However, Golgi proteins are still sensitive to *N*-glycosidase F, a glycosidase whose activity is not disturbed by the following carbohydrate modifications in the medial- and trans-Golgi. Calumenin had one putative *N*-linked glycosylation site. Affinity-purified FLAG-calumenin protein was incubated with either endoglycosidase H or *N*-glycosidase F and subjected to Western blotting with the anti-FLAG M2 antibody. We also performed the assay against FLAG-calumenin-rHDEL, whose C-terminal tetrapeptide HDEF was replaced with HDEL, known as the ER retention signal. Both FLAG-calumenin and FLAG-calumenin-rHDEL showed strong bands at 57 kDa without the glycosidases (Fig. 6, *lanes 1*, *3*, *5*, and *7*). Digestion with either endoglycosidase H or *N*-glycosidase F shifted the size of the strong bands to 52 kDa (*lanes 2*, *4*, *6*, and *8*). We consider that 57-kDa bands correspond to the glycosylated form of calumenin, and 52-kDa bands correspond to the deglycosylated form. Calumenin is indeed translocated and glycosylated in the ER and not modified in the Golgi apparatus, because Endo H completely cleaved oligosaccharides on FLAG-calumenin (*lanes 2* and *6*) and FLAG-calumenin-rHDEL (*lanes 4* and *8*). These results indicate that calumenin resides in the ER and that the C-terminal tetrapeptide HDEF works as the ER retention signal like HDEL (16, 17).

Expression and Chromosomal Localization of the Calumenin Gene—Northern blotting analysis showed that calumenin was expressed ubiquitously in all tissues examined. However, the expression of calumenin was strong especially in heart and lung. Compared with expression in adult heart, expression in 18.5-dpc heart was slightly stronger (Fig. 7*A*). The reverse transcriptase-PCR method showed that calumenin mRNA was already expressed as early as 8.5 dpc (Fig. 7*B*).

To determine the chromosomal localization of the calumenin gene (*Calu*),[2] strain distribution patterns of restriction fragment length polymorphisms of the calumenin gene were determined in 24 independent recombinant inbred strains derived from crosses between AKR/J and DBA/2J (AXD) (Table I). Analysis of the distribution pattern revealed the linkage of the calumenin gene with markers located at the proximal region of chromosome 7 (Fig. 8).

DISCUSSION

A Novel Ca^{2+}-binding Protein in the ER Lumen—We isolated and characterized a novel Ca^{2+}-binding protein calumenin from mouse embryonic heart. Calumenin is located in the ER and homologous to previously reported Ca^{2+}-binding proteins such as reticulocalbin and Erc-55 in the ER (16, 17), and Cab45 (18) in the Golgi apparatus. Since all of these proteins including calumenin have six EF hands and Ca^{2+}-binding activity, they constitute one subset of the EF-hand superfamily. A data base search also revealed that cDNA (λSCF13) cloned as DNA supercoiling factor of silkworm (43) also belongs to this family. The deduced amino acid sequence of λSCF13 has an amino-terminal signal sequence, six EF hands, and the C-terminal HDEF sequence, which we proved to be the ER retention signal.

Although many ER Ca^{2+}-binding proteins have been reported so far, their functions are largely not yet well understood. Two possible functions of ER Ca^{2+}-binding proteins have been suggested. First, calreticulin, one of the Ca^{2+}-binding proteins in the ER, is reported to regulate the capacity of Ca^{2+} in the ER (44, 45) as well as to have chaperone function (46, 47). Another luminal Ca^{2+}-binding protein, calsequestrin, is also reported to regulate Ca^{2+} flow from the ER (48, 49). These reports suggest that Ca^{2+} fluxes may be regulated somehow by

[2] The gene name *Calu* for calumenin has been approved by Lois J. Maltais, the Nomenclature Coordinator for the Mouse Genome Data base (The Jackson Laboratory, Bar Harbor, ME).

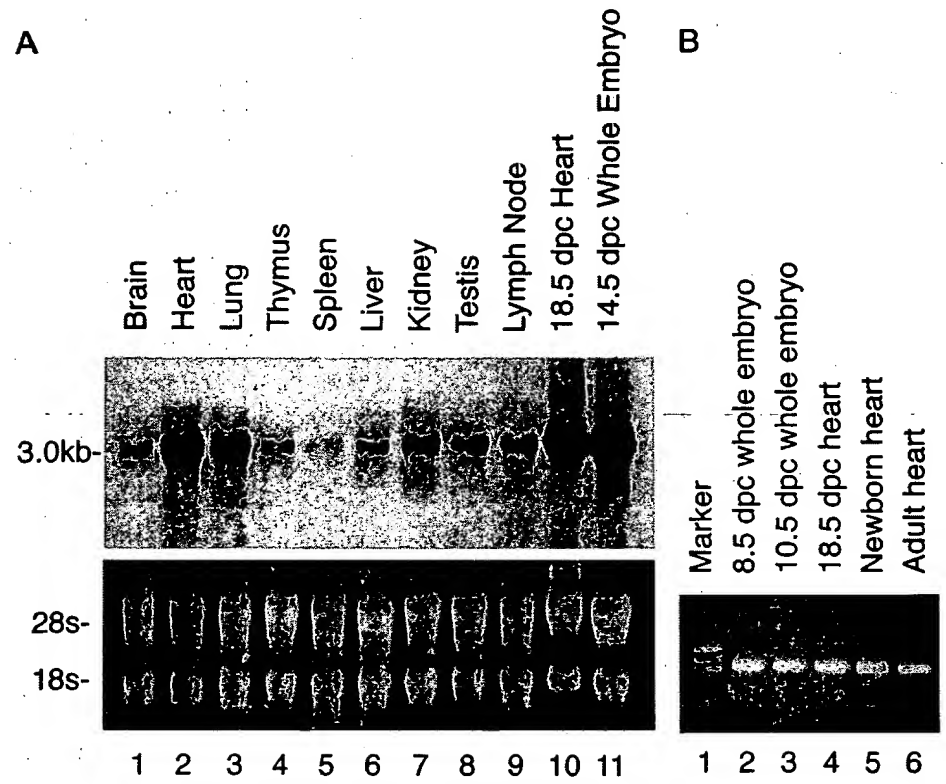


FIG. 7. **Expression pattern of calumenin.** *A*, Northern blotting of mouse RNA with calumenin cDNA as probe. 20 μg of total RNA from various mouse tissues were applied to each well (*lower column*), and Northern blotting was performed using a 512-base pair C39 fragment obtained in the initial screening as a probe (*upper column*). *B*, reverse transcriptase-PCR was performed using 1 μg of total RNA and calumenin-specific primers. After 28 cycles of PCR, products were electrophoresed and stained with ethidium bromide.

TABLE I

Strain distribution patterns of calumenin (Calu) and closely linked markers in RI strains AXD

Restriction fragment length polymorphisms were found by *Apa*I cleavage of the calumenin in AKR/J (3.8 kilobase pairs) and DBA/2J (4.8 kilobase pairs). Determination and analysis of the restriction fragment length polymorphism distribution pattern among recombinant inbred strains derived from AKR/J and DBA/2J were performed as described (35, 37). Strain-specific alleles are abbreviated as A (AKR/J) and D (DBA/2J). Unknown alleles that differ from A and D are identified with U. Putative crossing over points are indicated by X.

Locus	AXD recombinant inbred strain number																							
	1	2	3	6	7	8	9	10	11	12	13	14	15	16	18	20	21	22	23	24	25	26	27	28
Pmv4	A	D	A	A	D	D X	D	D X	D	D X	D	A	A	A	A	D	A	A	A	A	A	D	D	D
Calu	A	D	A	A	D	A	D	A	D	A	D	A	A	A	A	D	A	A	A	A	A	D	D	D
Emv11	A	D	A X	A	D	A	D	A	D	A	D X	A	A	A	A	D	A	A	A	A	A X	D	D	D
Ckmm	A	D	D	A	D	A	D	A X	D	A	A X	A	A	A	A	D X	A	A	A	A	D	D X	D	D
Pmv29	A	D	D X	A	D	A	D	D X	D	A	D X	A	A	A	A	A	A	A	A	A	D	A X	D	D
Mag	A	D	A	A	D	A	D	A	D	A	A	A	A	A	A	A	A	A	A	A	D	D X	D	D
Upk1a	A	D	A	A	D	A	D	A	D	A	A	A	A	A	A	A	A	A	A	A	U	A	D	D
Abpa	A	D	A	A	D	A X	D	A	D	A X	A	A	A	A	A	A	A	A	A X	A	D	A	D	D
Odc-rs6	A	D	A X	A	D	D	D	A	D	D	A	A	A	A	A	A	A	A	D	A	D	A	D	D
Xmv30	A	D	D	A	D	D	D	A	D	D	A	A	A	A	A	A	A	A	D	A	D	A	D	D
Tam1	A	D	D	A	D	D	D	A	D	D	A	A	A	A	A	A	A	A	D	A	U	A	D	D

Ca^{2+}-binding proteins located in the luminal side of the ER. Second, Ca^{2+} in the ER is reported to be necessary for normal functions of the ER such as protein folding and protein sorting (3–6), suggesting the existence of ER-resident molecules whose function is regulated by Ca^{2+}. In fact, Bip, a Ca^{2+}-binding protein in the ER, is reported to function in the Ca^{2+}-dependent manner (50).

Further study is needed to determine whether or not calumenin is involved in either of the two possible functions. Recently, interaction molecules of Erc-55 have been reported: the E6 protein of papilloma virus, which has p53-independent tumorigenic activity (51), and taipoxin, which blocks neuromuscular transmission at the presynaptic site (52). As discussed above, calumenin belongs to the same subset of the EF-hand superfamily as Erc-55. Thus, the search for proteins that interact with calumenin could provide a clue to elucidate biological functions of this subset.

A Novel ER Retention Signal—We report a novel carboxyl-

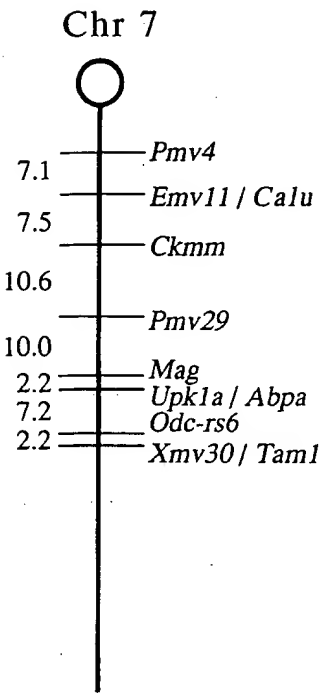


FIG. 8. **Chromosome map surrounding calumenin locus on mouse chromosome 7.** The position of the calumenin locus (designated by *Calu*) is shown on chromosome 7 based on data from AXD strains (Table I). The centromere is indicated by a *circle*. Recombination distances in centimorgans are shown at the *left* of the chromosome.

terminal tetrapeptide HDEF of calumenin as a new ER retention signal to prevent proteins from being secreted and to keep them in the ER. We observed that the HDEF sequence had the intracellular retention activity (Fig. 5). The deglycosylation assay (Fig. 6) and the immunostaining profile (Fig. 4) suggest that the HDEF sequence works as the ER retention signal. The C-terminal tetrapeptide KDEL is the first ER retention signal reported in mammalian cells (20). The C-terminal tetrapeptide HDEL, which was originally identified in yeast, was also found to work as the ER retention signal in mammalian cells (16, 17). Other reports show that the first and second positions of the C-terminal tetrapeptide are not strongly conserved to maintain the ER retention activity. In contrast, replacement of the third and fourth residues is strictly restricted. The third position requires an acidic residue such as Glu or Asp, and the fourth position requires a hydrophobic residue containing the aliphatic side chain such as Leu or Ile, which cannot be replaced by either Val or Ala (24, 25). Since Cab45, a soluble protein in the Golgi apparatus has HEEF sequence at the C terminus, this tetraresidue sequence was presumed to serve as the Golgi retention signal. The C-terminal Phe, a hydrophobic residue containing an aromatic side chain, was thought to work somehow differently from Leu (18). However, since our study clearly showed that the HDEF sequence worked as the ER retention signal, the retention signal to the Golgi apparatus may be localized in another portion of Cab45.

Proteins with ER retention signals such as KDEL are retrieved from Golgi. The convincing mechanism of this retrieval is that such proteins interact with the KDEL receptor distributing in the cis-Golgi and are retrieved to the ER (22, 23). We showed that the C-terminal HDEF sequence worked as the ER retention signal, but further investigation is needed to determine whether HDEF interacts with the KDEL receptor or with an unknown receptor.

Chromosomal Mapping of the Calumenin Gene (Calu)—The calumenin gene was mapped on the proximal portion of mouse chromosome 7. This region is well conserved between human and mouse, and the syntenic comparison suggests that this region corresponds to human 19q13.2–13.3 (53), at which the gene for progressive familial heart block type I, autosomal dominantly inherited cardiac bundle-branch disorder, is mapped (54). Since the causal gene of this disease has not been determined so far, further investigation is needed to determine whether the calumenin gene is involved in this disease.

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ANTIBODIES AGAINST A PEPTIDE SEQUENCE WITHIN THE HIV ENVELOPE PROTEIN CROSSREACTS WITH HUMAN INTERLEUKIN-2

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ABSTRACT

Recent investigations have identified a homologous sequence between the lymphokine interleukin 2 (IL-2) and the envelope protein of HIV. This homology is one of six amino acids corresponding to interleukin-2 (IL-2) residues 14-19 (Leu-Glu-His-Leu-Leu-Leu) and to the carboxy terminal six amino acids of HIV envelope protein gp41 (Leu-Glu-Arg-Ile-Leu-Leu). Thus, it is conceivable that an anti-HIV antibody response would generate antibodies which would crossreact with IL-2. We show here that the two peptides are recognized by the immune system as being almost identical. More importantly, antibodies against the HIV envelope peptide bind IL-2. Thus, these studies are the first step in investigating what could be characterized as an HIV-induced autoimmune response, that is, the induction of antibodies to the HIV envelope protein which also crossreact with IL-2.

INTRODUCTION

Recently, an amino acid sequence homology between the human immunodeficiency virus (HIV) envelope protein and human interleukin-2 (IL-2) was described (1,2). The homology is six amino acids in length and corresponds to the carboxy terminal six amino acids of the envelope protein, gp41 (3-5), and amino acids 14 to 19 of the secreted IL-2 molecule (6). For human IL-2, the sequence Leu-Glu-His-Leu-Leu-Leu (LEHLLL) shows a striking homology to a sequence in the HIV envelope protein Leu-Glu-Arg-Ile-Leu-Leu (LERILL). This six amino acid sequence is highly conserved among the viral strains isolated thus far (2-5;7-9), so there would seem to be some selective advantage for its presence.

One possible advantage might be the stimulation of an anti-LERILL antibody response upon viral infection which was crossreactive with IL-2. Since IL-2 is necessary for immune competence (10), antibodies against this protein might be deleterious. In fact, rabbit antisera against amino acids 7-21 of IL-2 (which includes LEHLLL) neutralized IL-2 biological activity (11). These studies along with others (1,12,13) demonstrate that the sequence LEHLLL is involved in binding of IL-2 to its receptor. Clearly, this sequence is important for the mediation of IL-2 biological activity.

The purpose of this study is to determine whether antibodies against the HIV envelope peptide, LERILL, recognize the IL-2 sequence, LEHLLL, and ultimately whether these antibodies will recognize the intact IL-2 molecule.

METHODS

Immunogen Preparation and Immunization

Each peptide was synthesized on a 9500 Solid Phase Peptide Synthesizer (Biosearch, San Rafael, CA) using t-boc chemistry (14). Peptides were cleaved from the resin using a hydrogen fluoride cleavage apparatus (Peninsula Laboratories, Belmont, CA) at 0°C in the presence of 10% anisole. After ethyl acetate precipitation, peptides were dissolved in 0.5 M acetic acid, lyophilized and then subjected to high pressure liquid chromatography (HPLC) purification using a C-18 150A Dynamax HPLC column (Rainin, Woburn, MA). Purity of peptides was assessed by the appearance of a single symmetrical peak on HPLC and amino acid compositional analysis. Peptides were greater than 98% pure using these criteria. Peptides were stored under nitrogen at 4°C until used.

For immunization, 5 mg of each peptide were coupled to 5 mg of keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA) using 10 mM glutaraldehyde in 0.05 M phosphate buffered saline (PBS, pH 7.2) for 4 hours at room temperature. After overnight dialysis against PBS, conjugates were aliquoted and stored at -20°C. Coupling efficiency was always between 65% and 80% as determined using the addition of ^{125}I-tyrosine containing analogs of each peptide to the coupling reaction to determine conjugation ratios.

Sprague Dawley rats (3 per group) were immunized subcutaneously and intraperitoneally with a total of 300 μg of peptide-KLH conjugate emulsified in incomplete Freund's adjuvant. Rats were boosted every 10 days for a total of four immunizations. Ten days after the final injection, rats were bled from the tail vein under ether anesthesia, and sera collected.

Enzyme-linked immunosorbant assay (ELISA)

To test antibody reactivity, peptides were adsorbed onto Nunc immuno-plates (Nunc, Thousand Oaks, CA) in 0.05 M carbonate buffer (pH 9.2) at 100 μg per well. After 18 hours at 4°C, nonadsorbed peptide was washed off with buffer (PBS-0.5% BSA-0.02% Tween-20) and the plates blocked for 2 hours with PBS-2% BSA. Sera or purified antibody was diluted in buffer, and varying dilutions were incubated on peptide coated plates for 90 minutes. After washing 3 times with buffer, a 1:1000 dilution of an anti-rat IgG antibody (Boehringer Mannheim, Indianapolis, IN) was added for 60 minutes. Unbound secondary antibody was removed by washing three times with buffer, and bound antibody was detected using nitrophenyl phosphate (Sigma, St. Louis, MO) in carbonate buffer (pH 9.2) as the substrate.

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Peptide Synthesizer
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Antibody reactivity was determined by reading the absorbance at 405 nm using a model 2550 EIA reader (Bio-Rad, Richmond, CA) approximately 30 minutes after substrate addition.

To assure specificity of binding, three different controls were used. First, sera from rats immunized with KLH only were used to demonstrate any nonspecific binding to the peptide. Second, binding of antisera to peptide coated wells was blocked with 1 mM soluble peptide. For blocking, peptides were conjugated to BSA via glutaraldehyde, and conjugation ratios were determined using ^{125}I-tyrosine containing derivatives of each peptide. Finally, irrelevant peptides (i.e., MAYKE and SATCTI) were coated onto plates to assess nonspecific binding of the antisera.

To test antibody reactivity against IL-2, the same ELISA procedure described above was used. Instead of peptides, recombinant IL-2 (AMGEN, Thousand Oaks, CA) was adsorbed onto Nunc high protein binding microwell modules (Nunc, Thousand Oaks, CA) at a coating concentration of 200 ng per well in carbonate buffer (pH 9.2). A heterologous rabbit anti-human IL-2 antibody (Genzyme, Boston, MA) was used for blocking experiments.

Affinity Purification of Antibodies

Antibodies to peptides were specifically purified using peptide conjugated affinity columns. Fifty mg of each peptide were dissolved in 0.05 M HEPES buffer (pH 8.0) and reacted with 10 ml of Affi-gel 10 (Bio-Rad, Richmond, CA) for 18 hours at 4°C. Unreacted groups were subsequently blocked with excess glycine. Sera from immunized rats were diluted 1:2 with PBS-0.05% aprotinin and passed over the appropriate affinity column. Bound antibodies were eluted with 0.1 M glycine-0.1 M NaCl (pH 3.0), subsequently neutralized and then dialyzed extensively against PBS. Antibody concentration was determined by absorbance at 280 nm (Shimadzu UV-160, Kyoto, Japan). Greater than 93% of protein binding to the affinity column was immunoglobulin as determined by densitometric scans of SDS-polyacrylamide gels.

RESULTS

Antibodies Against the HIV Envelope Peptide, LERILL, Also React with the IL-2 Sequence, LEHLLL

To demonstrate that antibodies against the HIV envelope peptide LERILL could also recognize the LEHLLL epitope of IL-2, groups of rats were immunized with either peptide. Figure 1 shows that when ELISA plates were coated with the peptide LEHLLL, antisera against LEHLLL specifically bound this peptide as would be expected. In fact, all three rats had titers to the peptide greater than 1:60,000. Controls demonstrated the specificity of this binding. Not only did anti-KLH sera not react, but greater than 90% of the antisera binding could be blocked with 1 mM soluble peptide conjugated to BSA. Irrelevant peptide-BSA conjugates blocked less than 8% of the binding. Furthermore, plates coated with irrelevant peptides reacted less than 5% as well as LEHLLL-coated plates.

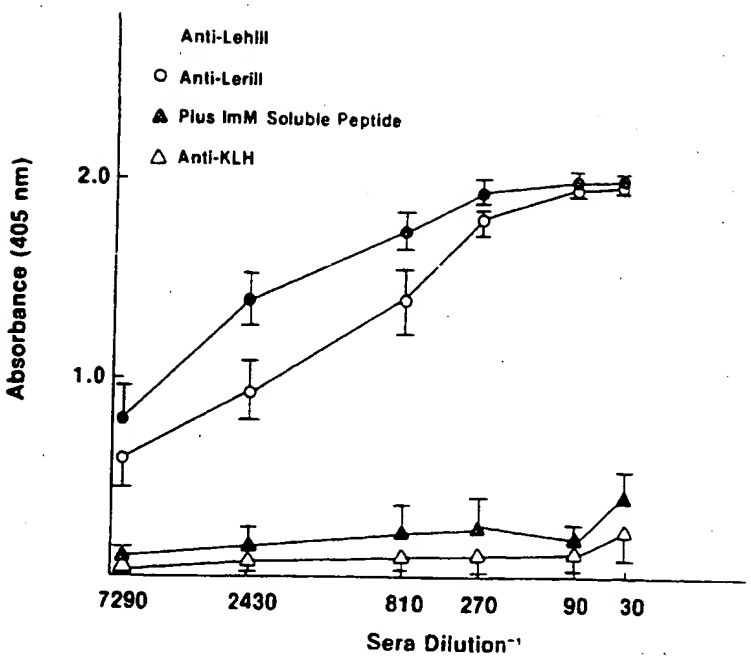


FIGURE 1

Reactivity of antisera against LEHLLL and LERILL on plates coated with LEHLLL. Sera from rats (3 per group) immunized with LEHLLL (●—●), LERILL (O—O) or KLH (Δ—Δ) were assayed for reactivity against LEHLLL using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM LEHLLL or 1 mM LERILL (▲—▲) to anti-LEHLLL sera during the reaction. Nonspecific binding is shown using anti-KLH sera. Each point represents the mean reactivity of three different rats ± standard deviations.

While it is clear that the ELISA reaction was specific, the important data in Figure 1 is the reactivity of sera from rats immunized with the HIV envelope peptide, LERILL, with the IL-2 peptide, LEHLLL. While the reactivity of antisera to LEHLLL is qualitatively higher, there is no statistically significant difference when antisera to LERILL is used. Furthermore, either soluble BSA-LEHLLL or BSA-LERILL could block greater than 90% of the binding.

Similar results were obtained when the reciprocal experiment was carried out. Plates coated with the HIV envelope peptide, LERILL, reacted specifically and with high titer (greater than 1:60,000) to sera from rats immunized with this peptide (see Figure 2). Once again, the reactivity using antisera against the homologue, LEHLLL, was not significantly different. Taken together, the results from Figures 1 and 2 conclusively demonstrate that the two peptides, LEHLLL and LERILL, are recognized by the

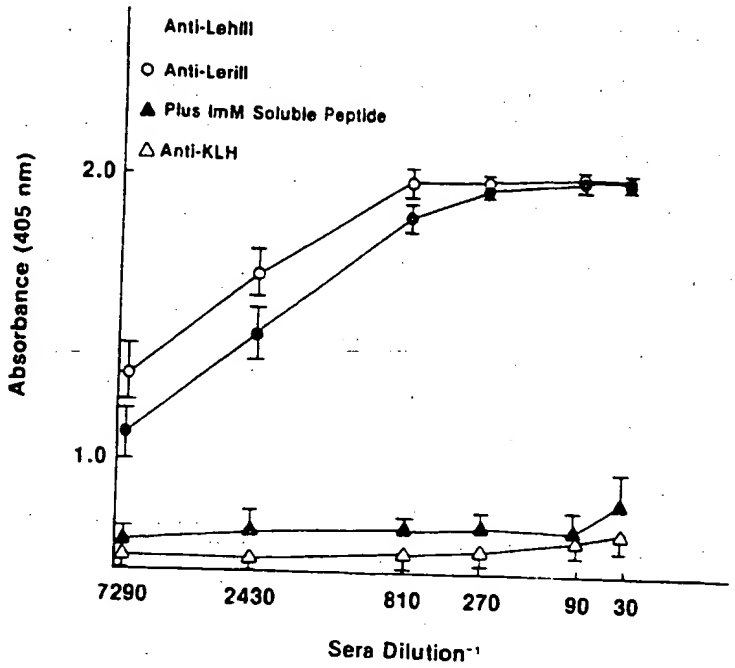


FIGURE 2

Reactivity of antisera against LEHLLL and LERILL on plates coated with LERILL. Sera from rats (3 per group) immunized with LEHLLL (●—●), LERILL (O—O) or KLH (Δ—Δ) were assayed for reactivity against LERILL using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM LEHLLL or 1 mM LERILL (▲—▲) to anti-LERILL sera during the reaction. Nonspecific binding is shown using anti-KLH sera. Each point represents the mean reactivity of three different rats ± standard deviations.

immune system as being almost identical. The ability of both antisera to recognize either peptide, and the ability to block both antisera reactivity with either peptide confirms this observation.

Anti-LERILL and anti-LEHLLL Antibodies React with IL-2

Since antisera against LERILL crossreacts with the IL-2 peptide, we questioned whether intact IL-2 would also be recognized. To demonstrate that antibodies specific for the HIV envelope peptide could react with IL-2, affinity chromatography was used. Each peptide was coupled to agarose beads, and the appropriate antisera was passed over each column. Figure 3 demonstrates the efficiency of the chromatographic procedure. Each purified antibody reacted with peptide, whereas the pass-through antisera did not. We concluded, therefore, that the affinity

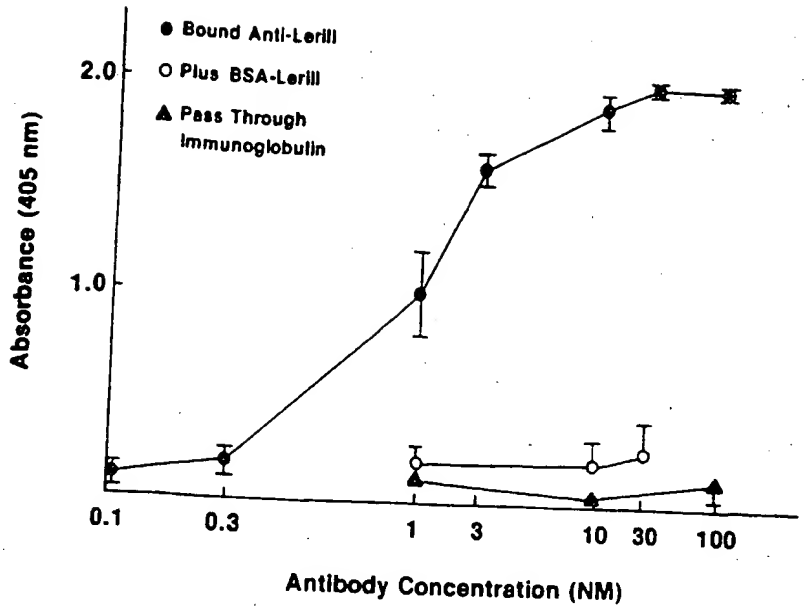


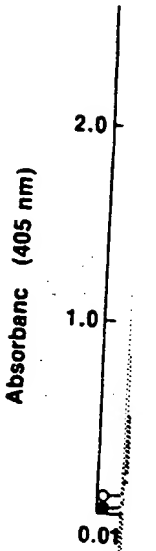
FIGURE 3

Reactivity of affinity purified anti-LERILL with LERILL. Sera from rats immunized with LERILL were passed over an agarose affinity column conjugated with LERILL. Antibody binding the column (●—●) and immunoglobulin which passed through the column (▲—▲) were assayed for reactivity against LERILL using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM soluble LERILL (O—O) to reactions involving antibody which bound to the column. Results are expressed as the mean of triplicate determinations ± standard deviations.

chromatography removed only the anti-peptide reactivity, since greater than 95% of the binding could be blocked with soluble peptide.

When IL-2 was coated onto plates, both anti-peptide antibodies reacted (see Figure 4). In fact, there was no significant difference between the ability of anti-LERILL or anti-LEHLLL antibodies to recognize IL-2. As little as 50 nM of either antibody could recognize IL-2. Thus, there is no doubt that specifically purified antibodies against the HIV envelope peptide, LERILL, react against IL-2.

If the IL-2 peptide, LEHLLL, is a significant epitope, then at least a portion of the antibodies raised against intact IL-2 should react with LEHLLL or its homologue, LERILL. To test this possibility, a rabbit anti-IL-2 antibody (Genzyme, Boston, MA) was allowed to react on IL-2 coated plates in the presence or absence of soluble peptide. Figure 5 demonstrates that either peptide could block approximately 25% of the heterologous antibody's binding. Considering that the epitope LEHLLL constitutes approximately 5% of the 15,500 dalton molecule weight of IL-2,



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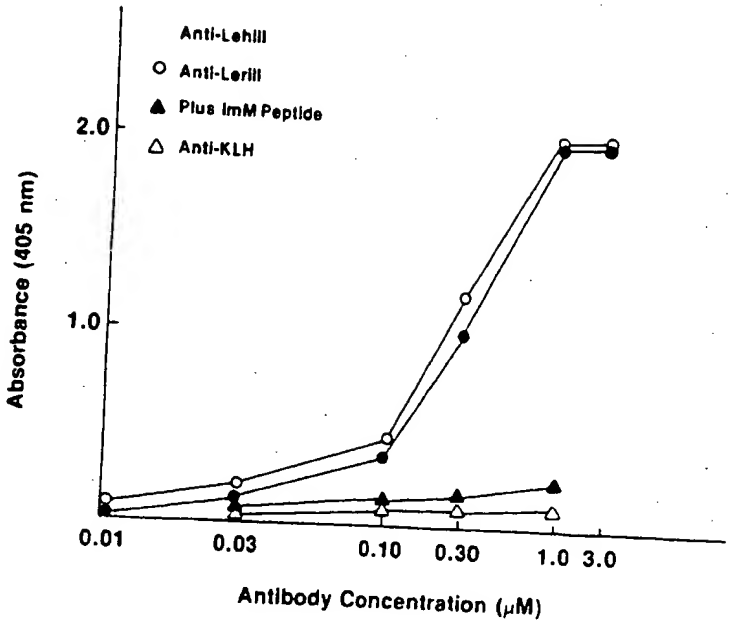

FIGURE 4

Reactivity of affinity purified anti-LEHLLL and anti-LERILL with IL-2. Affinity purified anti-LEHLLL (●—●), anti-LERILL (O—O), or anti-KLH (Δ—Δ) antibodies were assayed for reactivity against IL-2 using an ELISA. Specificity of the reaction was demonstrated by the addition of 1 mM LEHLLL or 1 mM LERILL (▲—▲). Results are plotted as means of triplicate determinations. Standard deviations were never more than ± 10% of the mean experimental value.

the percentage of antibodies reacting with this epitope is disproportionately high relative to its contribution to the molecular weight. Thus, LEHLLL would appear to be a major epitope on the IL-2 molecule. More importantly, the ability of the HIV envelope peptide LERILL to block anti-IL-2 antibody reactivity with IL-2 again supports the concept of this peptide being a homologue of LEHLLL.

DISCUSSION

We show here that antibodies raised against an HIV envelope peptide, LERILL, crossreact with human IL-2. The basis for this crossreactivity is the similarity of the HIV envelope peptide with its IL-2 homologue, LEHLLL. Not only does antisera against LERILL recognize its IL-2 homologue, LEHLLL, and vice versa, but either soluble peptide could block this binding (Figures 1 and 2). Anti-peptide antibody binding directly to IL-2 could be

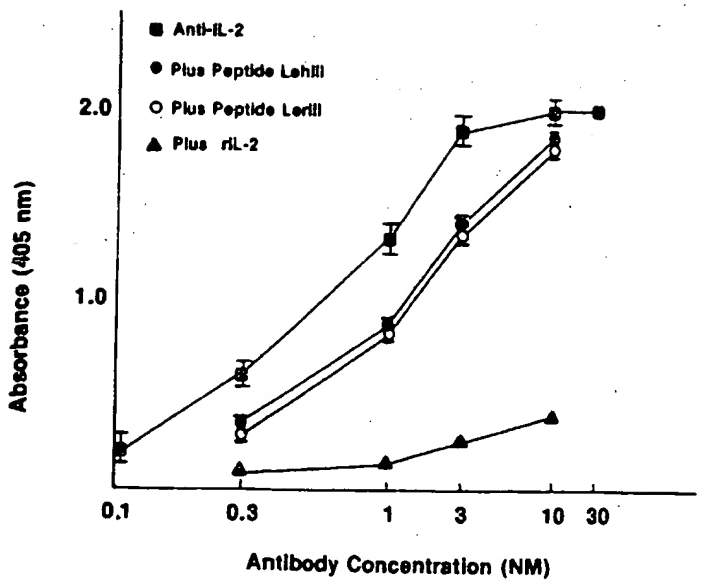

FIGURE 5

Inhibition of polyclonal anti-IL-2 binding by excess soluble LEHLLL or LERILL. Binding of a polyclonal anti-IL-2 antibody (■—■) was inhibited by 1 mM LEHLLL (●—●), 1 mM LERILL (O—O), or 1 μg per well recombinant IL-2 (▲—▲). Results are expressed as means of triplicate determinations ± standard deviations.

blocked by either soluble peptide as well (Figure 4). Thus, there can be no doubt that these two peptides represent crossreactive epitopes.

Establishing that the immune system recognizes these two peptide epitopes as being similar suggests a novel mechanism by which infection with HIV may contribute to the immunosuppression observed in this disease. Antibodies generated against IL-2 might be immunosuppressive since this lymphokine is of vital importance to immune competence (10). This possibility seems even more likely when one considers that the IL-2 sequence, LEHLLL, has been shown to be involved with IL-2 binding to the IL-2 receptor (1,12,13). If ongoing experiments in our laboratory do demonstrate that these crossreactive antibodies interfere with IL-2 mediated immune responses, then this would be a case where infection by a virus (i.e., HIV) results in the development of an autoimmune response (i.e., anti-IL-2 antibodies) by virtue of the presence of a crossreactive epitope present in the envelope protein.

In the majority of patients we have screened to date (15, manuscript in preparation), antibodies to the HIV envelope protein sequence, LERILL, have been detected. This finding may explain in part why serum IL-2 levels HIV-infected patients are low (16,17), and why IL-2 is needed to

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reconstitute some in vitro responses using lymphocytes from HIV-infected patients (18-22). Future experiments will be directed at determining the extent at which these antibodies contribute to the immunosuppression which is a hallmark of AIDS.

ACKNOWLEDGEMENTS

Special thanks are given to Diane Weigent for preparing the manuscript and to Todd McBurnett for expert technical assistance. This work was supported by NIAID grant AI25078.

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Vol. 43, No. 9, pp. 881–886, 1995
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Original Article

Possibilities of False Immunocytochemical Results Generated by The Use of Monoclonal Antibodies: The Example of the Anti-proinsulin Antibody[1]

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Received for publication December 19, 1994 and in revised form April 4, 1995; accepted April 26, 1995 (4A3559).

The immunocytochemical application of a monoclonal antibody (MAb) against the Arg-Arg region at the junction of the C-peptide and the insulin β-chain of the human proinsulin molecule on rat pancreatic tissue resulted in positive immunogold labeling over the insulin- as well as the glucagon-secreting cells. In the insulin cells, the Golgi cisternae and the immature clathrin-coated granules were intensely labeled, and in the glucagon cells all the granules were labeled. Control experiments using the anti-proinsulin MAb adsorbed with proinsulin, insulin, and glucagon have confirmed the crossreactivity of this proinsulin antibody with glucagon. Furthermore, the anti-proinsulin MAb appears to crossreact not only with rat but also with human glucagon and with bovine and porcine insulin and glucagon. Examination of the amino acid sequences of proinsulin and proglucagon has shown that both molecules display this Arg-Arg dipeptide sequence, which could explain the labeling obtained on both cell types. In addition, other propeptides also display such a sequence and, indeed, positive cells were detected in rat pituitary. These results demonstrate that by immunocytochemistry this MAb, although providing very specific results, reveals non-related molecules. This is due to the fact that these molecules exhibit similar short determinants. MAbs raised against very short amino acid sequences can reveal this determinant in various molecules, generating false-positive results. (*J Histochem Cytochem 43:881–886, 1995*)

KEY WORDS: Immunocytochemistry; Monoclonal antibodies; Insulin; Glucagon.

Introduction

The specificity of immunocytochemical results obtained at both the light and the electron microscopic level relies entirely on the properties of the first antibody, independent of the approach employed for the detection. Antibodies raised against a particular well-characterized antigen should be able to bind to this unique antigen and thus to discriminate it from all other proteins. This condition, which is a sine qua non for generating valuable results, has been somewhat challenged by the introduction of monoclonal antibodies (MAbs). Indeed, whereas polyclonal antibodies are composed of multiple species of immunoglobulins directed towards several determinants within a particular antigenic molecule, the MAbs are directed against single determinants consisting of very short sequences of amino acids (1). In this situation, although the MAbs are able to provide invaluable information about the molecular conformation of a particular determinant within that molecule, their use may in certain cases introduce some problems. In fact, although the specificity of the results is not affected, the multiplicity of molecules being recognized by a single MAb could be a problem. This is due to the existence of very similar determinants in various different peptides and proteins.

The aim of the present article is to report such a problem experienced with the use of a very well-characterized MAb directed against a very short determinant. Indeed, a particular MAb recognizing the region of the dipeptide Arg-Arg in the human proinsulin molecule (2) gives very specific and valid labeling in pancreatic insulin-secreting cells, revealing the sites of proinsulin molecules (3–5). However, because same or a similar determinant is also present in other peptides (6,7), the MAb generates positive labeling in other hormone-secreting cells, revealing not proinsulin but rather other non-related peptides. Concern is raised on this potential problem of getting false-positive cytochemical results by the use of certain MAbs.

Materials and Methods

For light microscopy, normal rat pancreatic tissue was fixed in Bouin's fixative and embedded in paraffin. For electron microscopy, human, rat, porcine, and bovine pancreatic tissues, as well as rat pituitary, were fixed by immersion with 1% glutaraldehyde and processed for embedding in Lowicryl K4M at −20°C (8). Paraffin sections were mounted on glass slides. Thin

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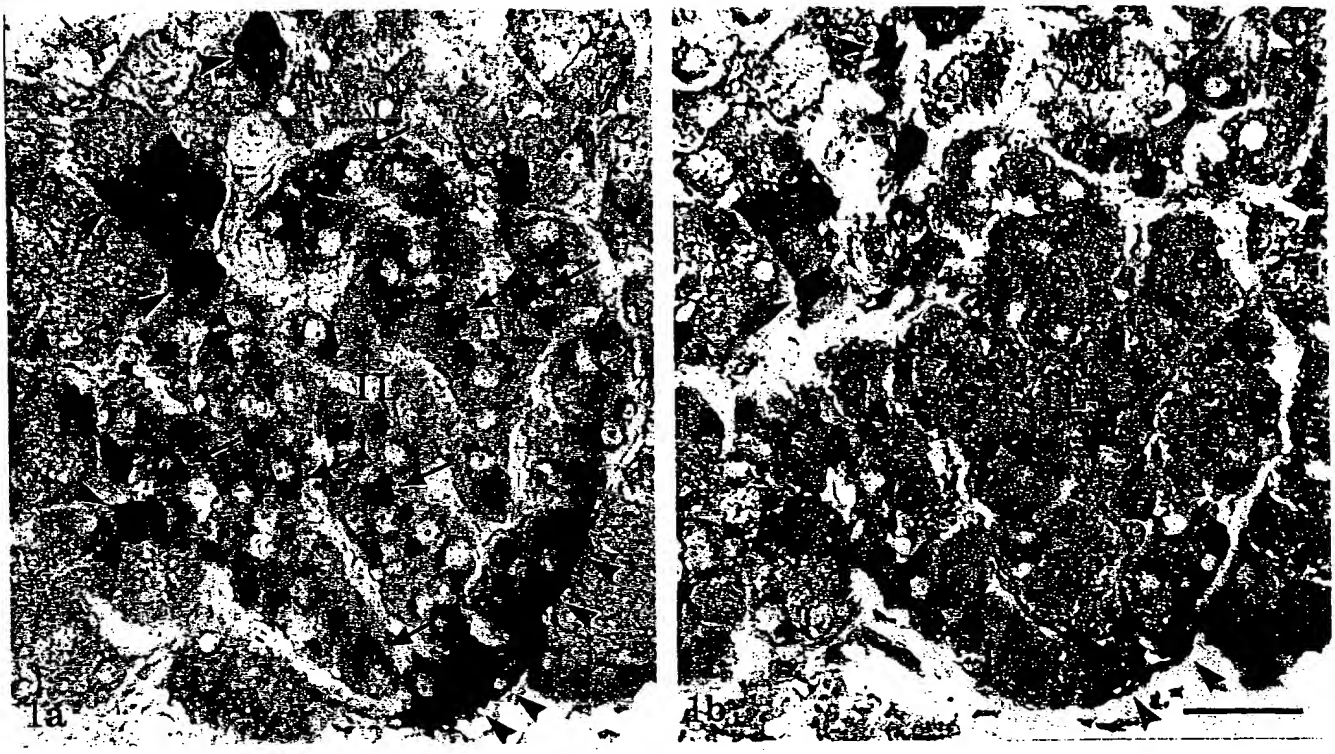


Figure 1. Light microscopic immunostaining on serial sections of an islet of Langerhans (IL) in rat pancreas. (a) Staining obtained with the anti-proinsulin MAb. (b) Staining obtained with the anti-glucagon antibody on a serial section. The anti-proinsulin MAb yields staining in the Golgi region (arrows) of the insulin-secreting cells in the central part of the islet. It also yields strong cytoplasmic staining in peripheral cells of the islets (arrowheads). On application of the anti-glucagon on a serial section (b), these same peripheral cells (arrowheads) display strong labeling. No staining was found on insulin-secreting cells with the anti-glucagon. Bar = 100 μm.

sections were mounted on nickel grids and processed for immunocytochemistry using a specific MAb and the protein A/G–gold or IgG–gold complexes.

The anti-human proinsulin MAb (Novo Nordisk BioLabs; Bagsvaerd, Denmark) used is directed against the region of the dipeptide Arg-Arg present at the junction between the C-peptide and the insulin β-chain within the human proinsulin molecule (2,9). It has been characterized by immunochemical and immunocytochemical techniques, having been used for specific demonstration of proinsulin molecules in rat pancreatic insulin-secreting cells (3,4). The anti-glucagon was a rabbit antiserum (INC; Stillwater, MN). The protein A/G–gold complex was prepared as reported previously with 10-nm gold particles (10); the IgG–gold complex was obtained from British BioCell (Cardiff, UK).

For light microscopic immunostaining, the sections were deparaffinized, rehydrated, and incubated with the MAb (dilution 1:10) for 2 hr at room temperature (RT). After rinsing with 0.01 M PBS, they were incubated with a secondary antibody, an anti-mouse IgG tagged with 10-nm gold particles, for 30 min at RT. The sections were further processed for the silver enhancement protocol (11) using the IntenSE M Silver Enhancement kit (Amersham Life Science; Poole, UK).

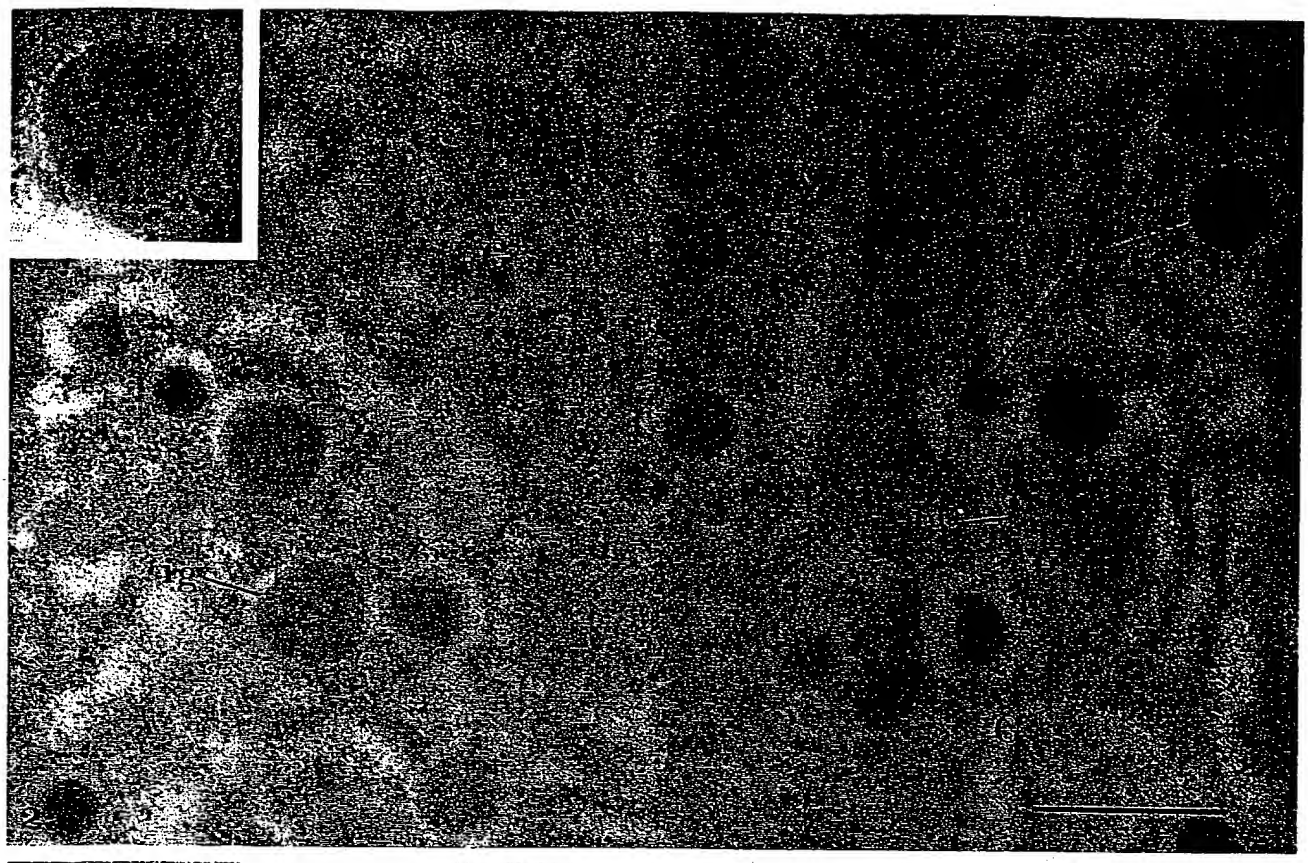
For electron microscopic immunolabeling, the thin sections were incubated with the same MAb (1:10 dilution) overnight at 4°C, followed by the protein A/G–gold complex for 30 min at RT. Sections were stained with uranyl acetate before examination with a Philips 410SL electron microscope.

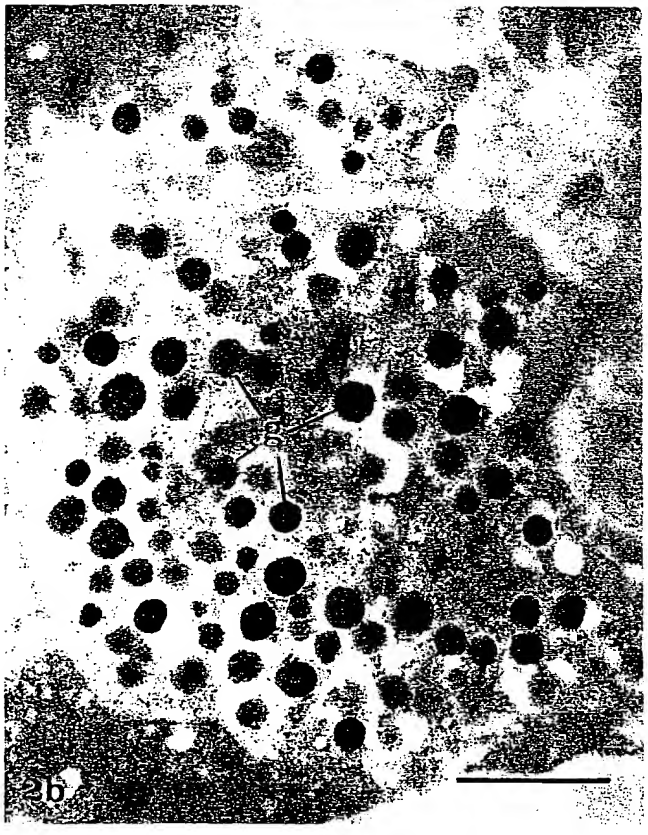
Control experiments were performed. Adsorption of the proinsulin MAb with different antigens was carried out before performance of the immunolabeling. Anti-proinsulin was adsorbed with insulin, proinsulin, and glucagon. The tissue sections were incubated with the adsorbed antibody and the protein A/G–gold complex according to the protocol described above.

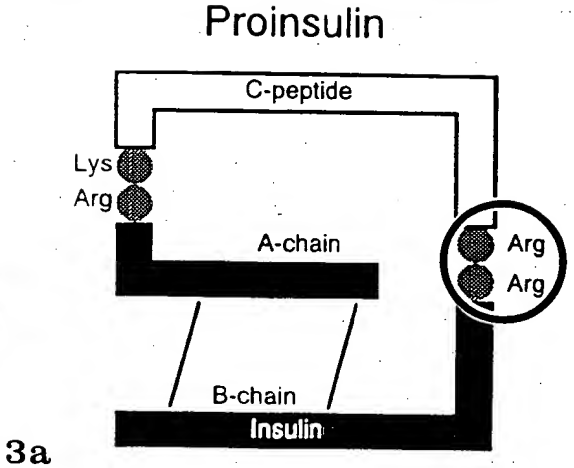
Results

By light microscopy, on application of the MAb against proinsulin, different cells of the islet of Langerhans in rat pancreas appeared labeled (Figure 1a). For the insulin-secreting cells located in the central part of the islet, the staining was not very intense but was concentrated in the Golgi area close to the nuclei. In contrast to this Golgi staining and in addition to the labeling of the insulin-secreting cells, other islet cells, few in number and located at the periphery of the islet, were also labeled and displayed very intense labeling involving the entire cell cytoplasm (Figure 1a). On applying the anti-glucagon antibody on a serial section, these same peripheral cells displayed positive labeling, demonstrating that they are in fact glucagon in nature (Figure 1b).

By electron microscopy on rat pancreatic tissue, the anti-proinsulin MAb yielded very specific labeling over the Golgi cisternae and over the light-cored immature secretory granules of the insulin-secreting cells (Figure 2a). In contrast, the mature insulin-containing granules, having a dense core, were not labeled. In several instances the labeled immature granules appeared delineated by a clathrin-coated membrane (Figure 2) as reported previously (3,4). In addition to labeling of the insulin-secreting cells, the







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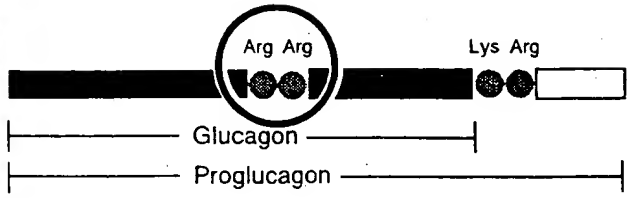

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Figure 2. Electron microscopic immunolabeling obtained with the anti-proinsulin MAb on rat pancreatic tissue. (**a**) In insulin-secreting cells, labeling is particula intense over the Golgi apparatus (G) and the immature secretory granules (ig). The mature granules (g) appear free of labeling. (**Inset**) A labeled immature grani (ig) displaying the typical clathrin-coated membrane (arrow). (**b**) A glucagon-secreting cell labeled with the same anti-proinsulin MAb, displaying strong labeli over the glucagon secretory granules (g). Bars: **a** = 0.5 μm; **b** = 1 μm.

Figure 3. The molecular conformation of (**a**) proinsulin and (**b**) proglucagon, showing that both molecules possess the dipeptide Arg-Arg.

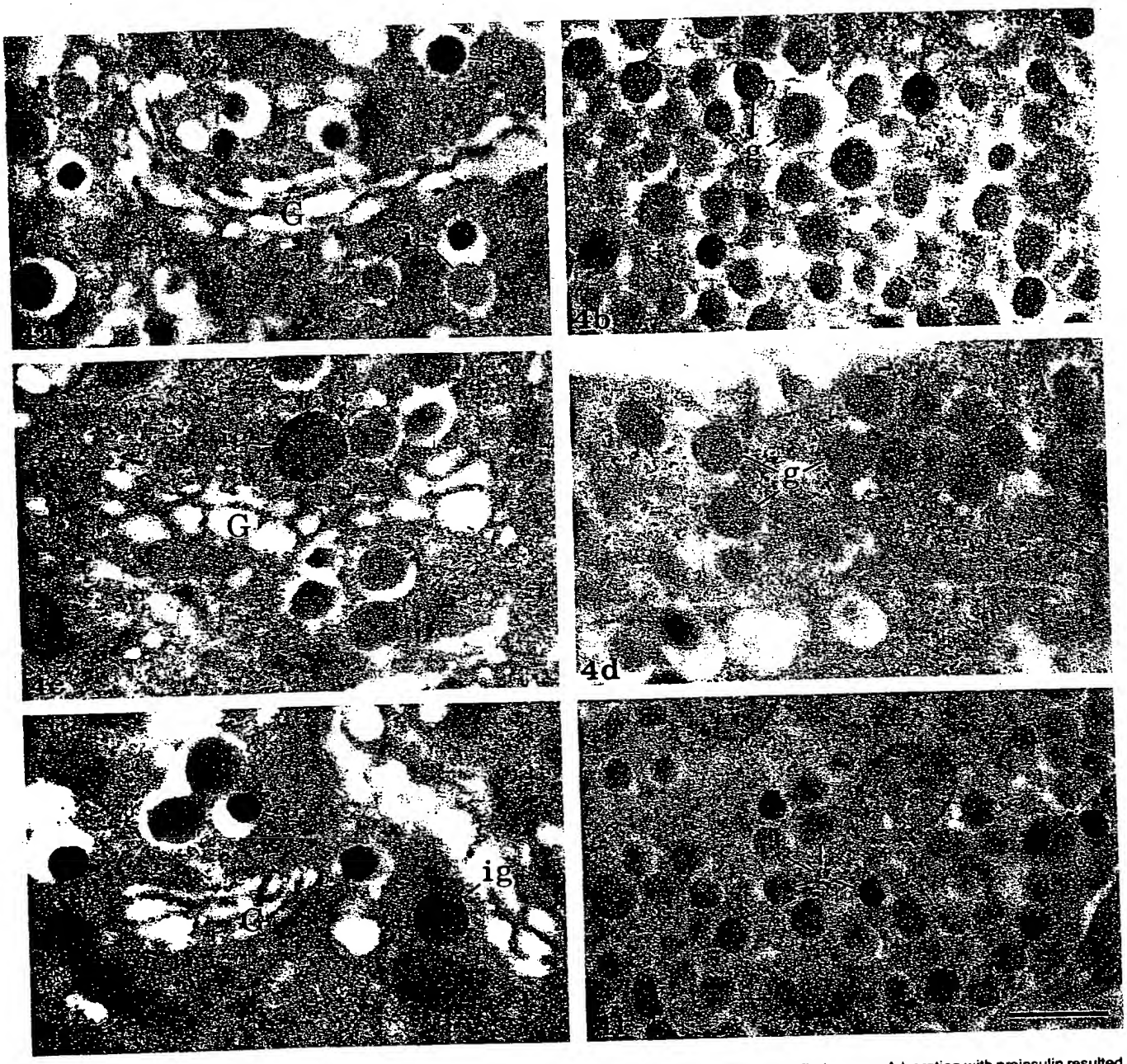

Figure 4. Control experiments. The anti-proinsulin antibody was adsorbed with (a,b) proinsulin, (c,d) insulin, and (e,f) glucagon. Adsorption with proinsulin resulted in complete elimination of the specific labeling in both the insulin (a) and the glucagon cells (b), whereas adsorption with insulin did not affect the intensity of labeling (c,d). For adsorption with glucagon, the intensity of labeling in insulin cells (e) was reduced, and that in glucagon cells was almost abolished (f). G, Golgi apparatus; ig, immature secretory granules; g, secretory granules. Bars = 0.5 μm.

glucagon-secreting cells, characterized by their dark granules having a very narrow clear halo and their peripheral location in the islet, were also labeled, displaying a significant number of gold particles over their secretory granules (Figure 2b). Somatostatin cells remained unlabeled (not shown).

Examination of the amino acid sequences of proinsulin and proglucagon molecules reveals similarities, with the existence of the dipeptide Arg-Arg in both molecules (Figure 3).

Control experiments using adsorbed anti-proinsulin were carried out with different antigens. When the anti-proinsulin was adsorbed with proinsulin, a drastic decrease in labeling intensity was observed in the immature secretory granules of the insulin cells, as well as in the glucagon secretory granules (Figures 4a and 4b). When it was adsorbed with insulin, no variation in the labeling pattern was detected in either type of cell (Figures 4c and 4d). Indeed, the immature secretory granules of the insulin cells and all the secretory granules of the glucagon cells remained intensely labeled. When the antibody was adsorbed with glucagon, a signifi-

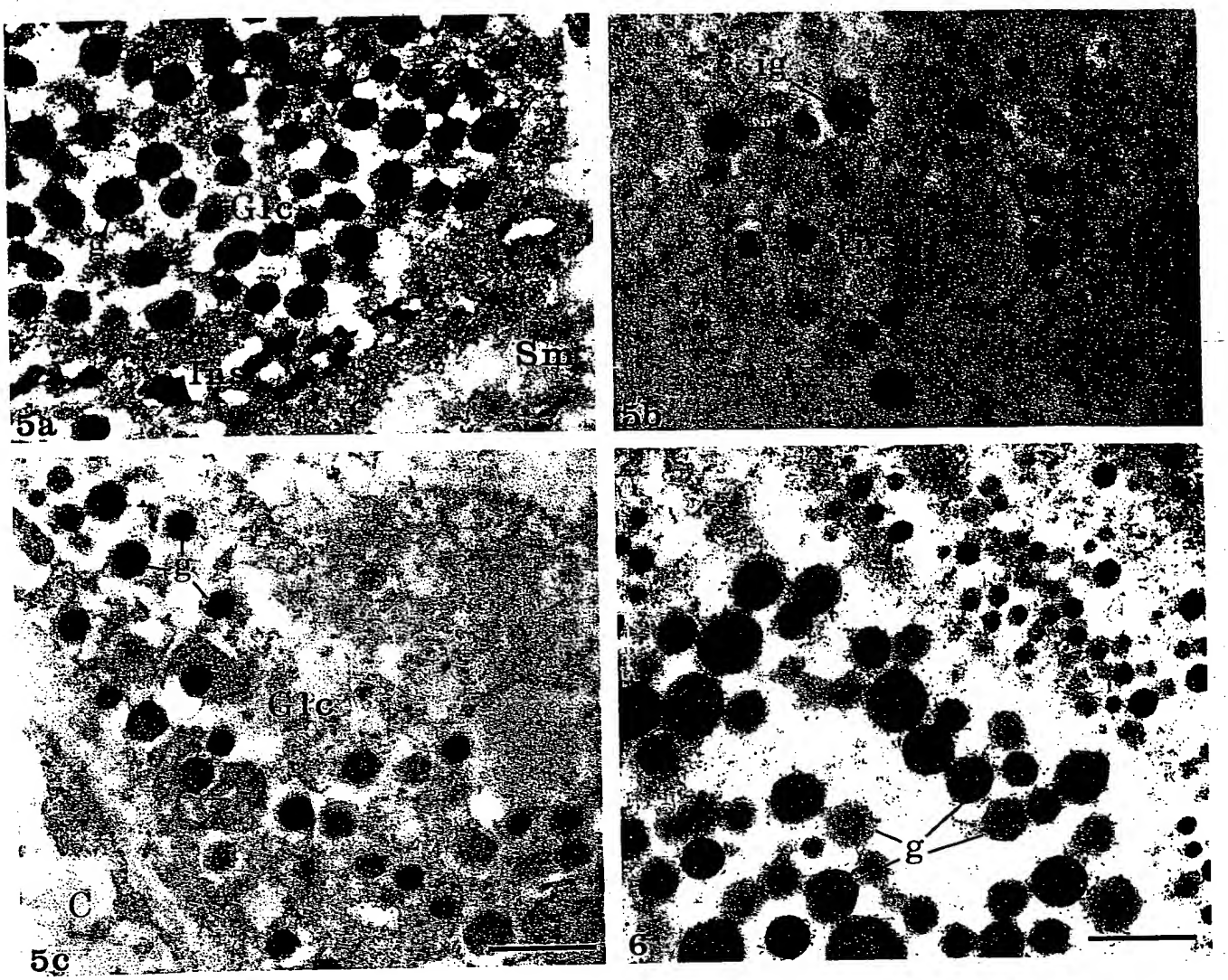

Figure 5. Immunolabeling obtained on (a) human, (b) bovine, (c) porcine insulin or glucagon cells with the proinsulin antibody. Most of the glucagon secretory granules in human and porcine cells (a,c), as well as some insulin secretory granules in bovine cells, probably immature ones (b), appear labeled by gold particles. Glc, glucagon cell; Ins, insulin cell; Sm, somatostatin cell; ig, immature granules; g, secretory granules; C, blood capillary. Bar = 0.5 μm.

Figure 6. Immunolabeling obtained on rat pituitary tissue with the proinsulin antibody. Some cells, characterized by their large dense granules (g), appear labeled by gold particles, whereas neighboring cells displaying small granules are devoid of labeling. Bar = 0.5 μm.

cant decrease in labeling was noted in the immature secretory granules of the insulin-secreting cells (Figure 4e), and the glucagon secretory granules were almost free of any labeling (Figure 4f).

Application of the anti-proinsulin antibody on human, porcine, and bovine pancreatic tissues resulted in specific immunolabeling on insulin- and on glucagon-secreting cells (Figure 5). For the insulin-secreting cells, labeling was restricted to a few secretory granules, which we assumed to be immature. The poor ultrastructural preservation, particularly on human tissue, did not permit identification of the clathrin coating of these granules. On the other hand, in glucagon-secreting cells most of the granules were labeled with the anti-proinsulin MAb, confirming the results obtained on the rat tissue (Figure 5). Somatostatin cells remained negative (Figure 5a).

Application of the anti-proinsulin MAb on tissue sections of rat pituitary revealed immunogold labeling over some endocrine cells (Figure 6). These cells were characterized by numerous large dark granules which displayed intense labeling. Neighboring cells containing small light granules were devoid of labeling (Figure 6).

Discussion

In the present study we used a very specific and well-characterized MAb raised against a very narrow and definite region in the human proinsulin molecule at the junction between the C-peptide and the insulin β-chain. This area comprises a particular dipeptide, Arg-Arg, which is the target of the convertase PC1 and which participates in the conversion of the proinsulin into C-peptide and

insulin (4,7,12). The MAb has been well characterized (2,9) and has been used in different studies for detection of proinsulin molecules within insulin-secreting cells (3–5). Indeed, confirming previous reports (3,4), application of this MAb with the immunogold approach on insulin-secreting cells led to labeling restricted to the Golgi cisternae and the immature clathrin-coated secretory granules (Figure 2). However, this same MAb gave a very defined and specific labeling over the glucagon-secreting cells, particularly at the level of the secretory granules. Analysis of the amino acid sequence of proinsulin and proglucagon molecules shows that the dipeptide region Arg-Arg is in fact present within both prohormone molecules (Figure 3). The presence of this determinant in the glucagon molecule could therefore explain the specific and strong labeling found in the glucagon-containing granules, as well as in the results obtained by the control experiments.

By immunochemical techniques, this proinsulin MAb was shown to recognize the Arg-Arg region of the human proinsulin molecule and not to cross-react with bovine or porcine proinsulin (2,9). However, by immunocytochemistry, this same MAb appears not only to react with human proinsulin but also to crossreact with porcine, bovine, and rat proinsulin. In addition, it also binds glucagon molecules in pancreatic cells from all of the above-mentioned species. Moreover, it also appears to recognize some rat pituitary hormones, probably the pro-opiomelanocortin, which displays similar Arg-Arg regions (6). Although no biochemical test of crossreactivity was performed with other prohormones, the immunochemical techniques have assigned a very narrow specificity to this antibody (2,9). The wider crossreactivity displayed by this antibody when used with morphological means could be based on the concept proposed (9) that the antibody recognizes not only a very particular amino acid sequence but also the three-dimensional conformation of this determinant. The processing of the tissues for morphological studies, which is known to affect the structure of proteins, may favor particular conformation(s) of similar determinants to resemble the domain present in the parent molecule.

Short amino acid sequences of identical nature can be present in various proteins, and an antibody directed against such a sequence, although still yielding specific labeling, could reveal different molecules not related to the original antigen. This problem, already discussed by Mason et al. (13) and illustrated by the present results, raises the possibility of false-positive labelings with monoclonal antibodies and stresses the care that should be taken before any rapid interpretation of immunocytochemical results.

Acknowledgments

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Effects of Amino Acid Substitutions Outside an Antigenic Site on Protein Binding to Monoclonal Antibodies of Predetermined Specificity Obtained by Peptide Immunization: Demonstration with Region 94–100 (Antigenic Site 3) of Myoglobin

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Amino acid substitutions outside protein antigenic sites are very frequently assumed to exert no effect on binding to antiprotein antibodies, especially if these are monoclonal antibodies (mAbs). In fact, a very popular method for localization of residues in protein antigenic sites is based on the interpretation that whenever a replacement causes a change in binding to antibody, then that residue will be located in the antigenic site. To test this assumption, mAbs of predetermined specificity were prepared by immunization with a free (i.e., without coupling to any carrier) synthetic peptide representing region 94–100 of sperm whale myoglobin (Mb). The cross-reactivities and relative affinities of three mAbs with eight Mb variants were studied. Five Mb variants which had no substitutions within the boundaries of the designed antigenic site exhibited remarkable, and in two cases almost complete, loss in cross-reactivity relative to the reference antigen, sperm whale Mb. Two myoglobins, each of which had one substitution within region 94–100, showed little or no reactivity with the three mAbs. It is concluded that substitutions outside an antigenic site can exert drastic effects on the reactivity of a protein with mAbs against the site and that caution should be exercised in interpreting cross-reactivity data of proteins to implicate residues directly in an antigenic site.

KEY WORDS: Amino acid substitutions; monoclonal antibodies; myoglobin; predetermined specificity; synthetic antigenic site.

1. INTRODUCTION[3]

Immunochemical cross-reactions of protein mutants are very often used to implicate the involvement of certain amino acid residues on a protein in its antigenic sites. The data are interpreted on the basis of the assumption that every amino acid replacement on the antigen which results in changes in its binding properties is directly involved in the interaction with antibody, particularly when a monoclonal antibody (mAb) is used (Berzofsky *et al.*, 1980, 1982, 1983; van Regenmortel, 1988). On the other hand, it has been shown that substitutions outside protein antigenic sites will influence the antigenic reactivity of the site because of one or more of the following factors: conformational and steric readjustments, electron delocalization, charge redistribution, and changes in hydrogen bonding or hydrophobic interactions (Kazim and Atassi, 1980; Atassi and Kazim, 1980;

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[3] Abbreviations used: BSA, bovine serum albumin; mAb, monoclonal antibody; mAb C_{50}, monoclonal antibody concentration at 50% cross-reactivity; Mb, myoglobin and when preceded by an abbreviation it denotes the following Species: Bg, badger, Ch, chicken; Cs, California sea lion; Dg, dog; Ed, echidna; Fb, finback whale; Pd; pacific common dolphin; Sp, Sperm whale; PBS, 0.15 M Nacl in 0.01 M sodium phosphate buffer, *p*H 7.2; RIA, radioimmune assay.

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Twining *et al.*, 1981). These latter studies were done with polyclonal antibodies whose recognition was directed at a number of antigenic sites in each multideterminant protein antigen [five in Mb (Atassi, 1975) and three in lysozyme (Atassi, 1987)]. Therefore, the assignment of the effects of substitutions on a given site was difficult to determine and could only be estimated from their distances in the three-dimensional (3D) structure from the site residues. Clearly, to resolve this issue, mAbs against a single site are needed whose specificity is known precisely and which is not deduced by the very approach it is set out to test.

To determine in an unequivocal manner whether the reactivity of an antigenic site is influenced by substitutions outside the site, we have employed here monoclonal antibodies that were generated by immunization with a synthetic peptide representing region 94-100 [antigenic site 3 (Atassi, 1975)] of SpMb. The peptide was used as an immunogen in its free state (i.e., without coupling to any carrier) to produce mAbs of predetermined specificity (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*; for review, see Atassi and Young, 1985; Atassi, 1986). Cross-reactions of Mbs which do not have substitutions in this region revealed that substitutions outside the site can exert dramatic effects on the binding of a protein with anti-site mAbs.

2. MATERIALS AND METHODS

2.1. Materials

The isolation and purification of myoglobins of various species were performed by previously reported procedures (Atassi, 1964, 1970; Twining *et al.*, 1980). BgMb, CsMb, and PdMb were obtained from Dr. C. R. Young (Texas A&M University). The homogeneity of each Mb was confirmed by polyacrylamide-gel electrophoresis. The synthetic peptide, Ala-Thr-Lys-His-Lys-Ile-Pro, corresponds to antigenic site 3 (residues 94-100) of SpMb (Atassi, 1975). Synthesis, purification, and characterization of the peptide have been described previously (Pai and Atassi, 1975). The synthetic peptide was used as an immunogen *in its form* (i.e., without coupling to any carrier) in Balb By J mice (6-8 weeks). The mice were injected and boosted biweekly with 25 μg of peptide [previously found (Young *et al.*, 1983) to be the optimum dose in this strain] as an emulsion of a solution in PBS (50 μl) with an equal volume of Freund's complete adjuvant. The mice received boosters until good antibody titers against the peptide and SpMb were obtained in the sera. Monoclonal antibodies were prepared as described (Schmitz *et al.*, 1982, 1983*a*, *b*). Sera and culture supernatants were screened for hybridoma antibodies by a solid-phase RIA on poly(vinylchloride) plates (Costar, Cambridge, Massachusetts) using the peptide-BSA conjugate and SpMb as the plate antigens (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*).

2.2. Determination of PVC-Plates Capacity for Various Myoglobins

It was necessary to rule out the possibility that any differences that might be found in the binding of mAbs to the various Mbs will not be caused by differences in the amounts of Mbs bound to the plates. Therefore, the amount of each Mb required to saturate the plate was determined. Each protein (25 μg) was labeled with ^{125}I by the chloramine-T method (Hunter, 1969). A fixed amount of each Mb (5×10^5 cpm) was mixed with the respective unlabeled Mb (25 μg). Serial dilutions were made and 50 μl aliquots of each dilution were plated in triplicate into the wells of the plate. The plates were incubated at 37°C for 3 hr, after which they were washed six times with PBS, dried, cut out, and counted in gamma counter. The amount of each labeled Mb bound (in cpm) was plotted against its concentration. From these plots, it was possible to determine, for each Mb, the concentration needed to saturate the well.

2.3. Determination of Cross-Reactivities and Relative Affinities

Cross-reactivity studies were done as described by East *et al.*, (1982) and Leach (1983). In the present work, pvc plates were incubated (37°C, 3 hr) with saturating amounts (50 μl of 50 μg/ml) of various myoglobins after which they were blocked (37°C, 1 hr) with 100 μl of 1% BSA in PBS. Aliquots (50 μl) of serial dilutions of each mAb (in 0.1% BSA in PBS) were added to the wells and the plates were incubated at room temperature for 5 hr and then washed (five times) with PBS. The second antibody (rabbit anti-mouse IgMk), appropriately diluted with PBS-0.1 BSA, was added (50 μl) and the plates were incubated at 37°C for 2 hr. The plates were washed six times with PBS and then ^{125}I-labeled protein A (2×10^5 cpm) was added in 50 μl of PBS-0.1% BSA. Following incubation at room temperatue for 2 h, the plates were

washed with PBS and dried. The wells were then cut out and their radioactivity measured in a gamma counter. Nonspecific binding was calculated from the binding of antipeptide mAbs (the test antibodies) to wells coated with BSA. Additional negative controls included the binding, to each test Mb, of normal mouse and human IgM and culture supernatant from P3 × 63-Ag8-653 myeloma cell lines. The results were expressed in percent cross-reactivity relative to antibody bound to SpMb as 100%. Measurements of relative binding affinities were determined by antibody dilution analysis (van Heyningen, 1986). Serial dilutions of mAb were allowed to bind to each Mb as described above. Affinities were ranked on the basis of the concentration of antibody required to give 50% of maximum binding (C_{50}) relative to the C_{50} with SpMb as 1.00. This relative ranking of affinities is valid only for mAbs which bind to a single determinant on the antigen used for dilution analysis, which is the case here. Concentrations of mAb solutions were determined by calibrated solid-phase RIA relative to standard curves of known concentrations of affinity-purified mouse IgMk.

2.4. Calculation of the Distance between Site Residues and All the Other Mb Residues

The C^{α}-to-C^{α} distances as well as the atoms of nearest approach between each of the residues 94 through 100 (antigenic site 3) of SpMb and all other residues in the molecule were calculated, from the SpMb coordinates at 2.0 Å (Takano, 1984), on a VAX 8550 using the program chain (Sacks, 1988). These distances had previously been calculated (Kazim and Atassi, 1980) but subsequent refinement of Mb structure (Takano, 1984) necessitated the recalculation of these distances.

3. RESULTS

3.1. Binding of the Myoglobins to the Plates

It was necessary to ensure at the outset that the differences in the antigenic reactivities of the various Mbs were not caused by differences in the amounts of each Mb bound to the plates. The results (Fig. 1) using ^{125}I-labeled Mbs showed that each Mb was able to saturate the well when its concentration was 25 μg/ml (1.5 μg/well). Therefore, in all subsequent studies, the Mb solutions employed 50μg/ml (2.5 μg/well), which is twice the concentration necessary to saturate the wells.

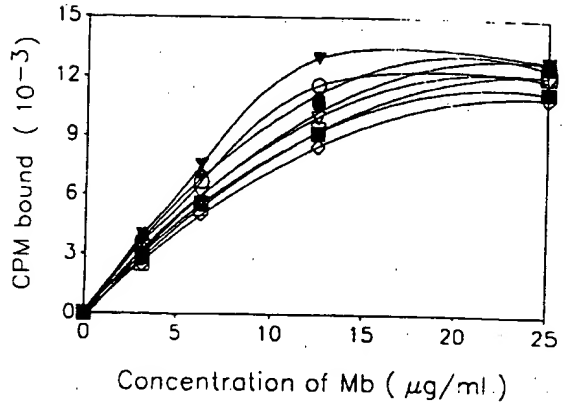

Fig. 1. Determination of PVC-plate binding capacity to myoglobin variants. The binding capacity of the wells of PVC plates for the various myoglobins was determined using increased amounts of ^{125}I-labeled Mb and unlabeled Mb: (○) BgMb, (■) ChMb, (◆) CsMb, (▼) DgMb, (▽) EdMb, (◇) FbMb, (□) PdMb, and ( ) SpMb. For experimental details, see the text.

3.2. Characterization of Antipeptide Antibodies

Pre-immune sera from the mice showed no antibody binding to the immunizing peptide 94–100 or to SpMb. The mice were injected with the optimum dose (25 μg/mouse) of peptide 94–100 and boosted until the sera possessed high titers of antibodies that bound to the peptide and to SpMb. The polyclonal antisera and the mAbs obtained from the mouse B cells after hybridization were entirely specific to the peptide and SpMb. They did not react with any SpMb peptides or any other proteins and peptides (from our large library of synthetic peptides) that are not related to the Mb system. The specificity of peptide-generated mAbs was in agreement with previous reports (Schmitz *et al.*, 1983*a*, *b*; Atassi and Young, 1985).

A panel of 18 mAbs giving high levels of binding to SpMb was prepared from fusion of spleen cells (6 mAbs) and of lymph node cells (12 mAbs). All the mAbs (as well as the polyclonal antisera) were of IgM class and *k* light chain. Three mAbs (nos. 708, 718, and 720) were used for this work on the basis of their higher relative-binding affinities

3.3. Binding of the Monoclonal Antibodies to the Various Myoglobins

Of the myoglobins prepared for those studies, five have no substitutions within the region 94–100 (to which the antibody specificity is directed) and two have each one substitution in the site (Table I).

Table I. Antigenic Site 3 of Sperm Whale Mb and Substitutions Within This Region in Other Myoglobins[a]

Myoglobin	Residue no.	94	Site 3 95	96	97	98	99	100
Sperm whale		Ala	Thr	Lys	His	Lys	Ile	Pro
Finback whale								
Pacific common dolphin								
California sea lion								
Dog								
Chicken								
Echidna								Ser
Badger							Val	

[a] The amino acid substitutions were based on the sequences given in the references cited: sperm whale Mb (Edmundson, 1965; Romero-Herrera and Lehmann, 1974); finback whale Mb (DiMarchi *et al.*, 1978); Pacific common dolphin Mb (Wang *et al.*, 1977); California sea lion Mb (Vigna *et al.*, 1974); dog Mb (Dumur *et al.*, 1976); chicken Mb (DeConinck *et al.*, 1975; Romero-Herrera *et al.*, 1978); echidna Mb (Castillo *et al.*, 1978), and badger Mb (Tetaert *et al.*, 1974).

The myoglobins that have no substitutions within region 94–100 are CsMb, FbMb, PdMb, ChMb, and DgMb. The cross-reactions of these myoglobins with antipeptide 94–100 mAbs 708, 718, and 720 were determined as a function of mAbs concentration. The results of binding to CsMb, FbMb, and PdMb are shown in Fig. 2, while Fig. 3 summarizes the results with ChMb and DgMb. With a given mAb, the cross-reactivity of each of these five myoglobins was markedly decreased relative to the reference antigen, SpMb. The extents of maximum cross-reactions (relative to SpMb as 100%) of CsMb, FbMb, and PdMb with mAbs 708, 718, and 720 in that order were CsMb, 47%, 35%, and 43%; FbMb, 47%, 37%, and 64%; PdMb, 39%, 40%, and 61%. In the case of ChMb and DgMb, their cross-reactions were dramatically and unexpectedly low (less than 20%, Fig. 3).

For the two myoglobins of eschidna and badger, each has one substitution within region 94–100 (Table I). These two myoglobins showed little or no reactivity with the three mAbs (Fig. 4).

Expression of the results in terms of the relative-binding affinities (Fig. 5) showed that all seven myoglobins (including the five that have no substitutions within the boundaries of the site) had greatly reduced binding affinities, relative to SpMb. With only one mAb (no. 720), two myoglobins that have no substitutions in the site (PdMb and FbMb) showed relative affinities that were measurable by this method, and even these were much lower (70%) than that of SpMb with this mAb.

4. DISCUSSION

It was found relatively early that the reaction of a protein antigen with its antibodies is influenced by conformational changes (Atassi, 1967, 1970; Habeeb and Atassi, 1971). The first clear evidence of this was in fact obtained with Mb and hemoglobin. In these two proteins, even though the heme group is not part of an antigenic site (Reichlin *et al.*, 1963; Atassi, 1967), derivatives whose conformation was intentionally altered using hemes with modified side chains or metal substitutions (to replace the iron) exhibited remarkable changes in antigenic reactivity (Atassi, 1967; Atassi and Skalski, 1969; Andres and Atassi, 1970). Subsequent findings with other protein systems (Atassi *et al.*, 1970; Habeeb and Atassi, 1971; Arnon and Maron, 1971, Arnheim, 1973; Prager *et al.*, 1974; Atassi and Habeeb, 1977; Habeeb, 1977) made it evident that the antibody response against native protein antigens is directed against their native three-dimensional structure (Atassi, 1967, 1975, 1978, 1984). This is now a well-established principle in molecular immunology.

Amino acid substitutions can cause conformational readjustments which will influence antigenic reactivity (Atassi, 1970; Atassi *et al.*, 1970; Arnheim, 1973, Prager *et al.*, 1974). Quite often, however, amino acid substitutions do not induce any detectable conformational changes but will nevertheless influence antigenic reactivity. The replacements may result in electron delocalization and charge redistribution and/or changes of hydrogen bonding and hydrophobic interactions which could alter the binding properties of an antigenic site. Indeed, studies with Mb (Kazim and Atassi, 1980; Twining *et al.*, 1980) and lysozyme (Atassi and Kazim, 1980) showed that substitutions outside the antigenic sites of the respective protein (Atassi, 1975, 1978) exerted remarkable effects on its antigenic reactivity, and it was concluded that not every replacement that causes a change in

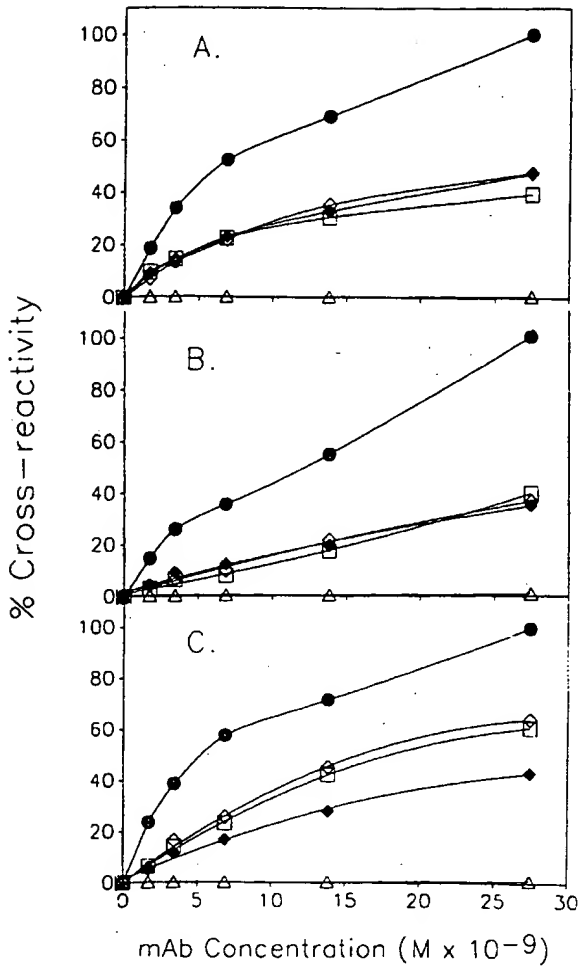

Fig. 2. Cross-reactivity of antisite 3 mAbs with PDMb, FbMb, and CsMb, which have no substitutions in this site. This binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentration, to SpMb (●), PdMb (□), FbMb (◇), and CsMb (◆) was determined. The microtiter wells were coated with 50 μl of Mb solutions having the same protein concentration (50 μg/ml) and the assays were done as described under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to the maximum bound by SpMb as 100%) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, PdMb, FbMb, and CsMb.

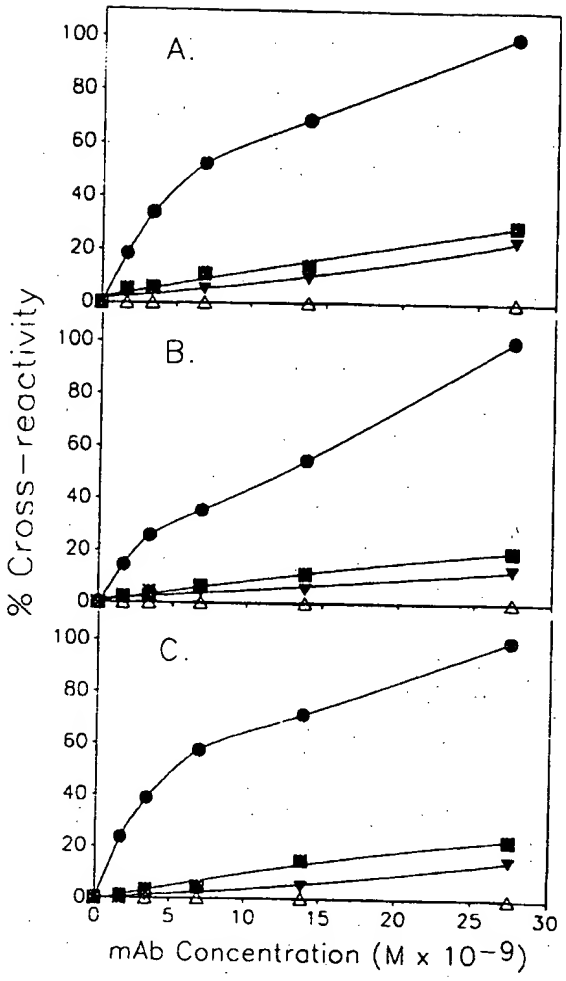

Fig. 3. Cross-reactivity of antisite 3 mAbs with ChMb and DgMb which have no substitution in that site. The binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentration, to SpMb (●), ChMb (■), and DgMb (▼) was determined. The microtiter wells were coated with (50 μl of Mb solutions having the same antigen concentration (50 μg/ml)). The assays were done as described under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to the maximum bound by SpMb) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, ChMb, and DgMb.

antigenic reactivity will necessarily reside in an antigenic site.

On the other hand, others have preferred to ascribe a direct role for all replacements that influence reaction of antigen with antibody, particularly when the latter is a monoclonal antibody. Thus, it has been clearly stated that "amino acid substitutions which affect binding by monoclonal antibodies are most likely to be within the antigenic site than far from it" (Berzofsky *et al.*, 1982). More recently, it was similarly

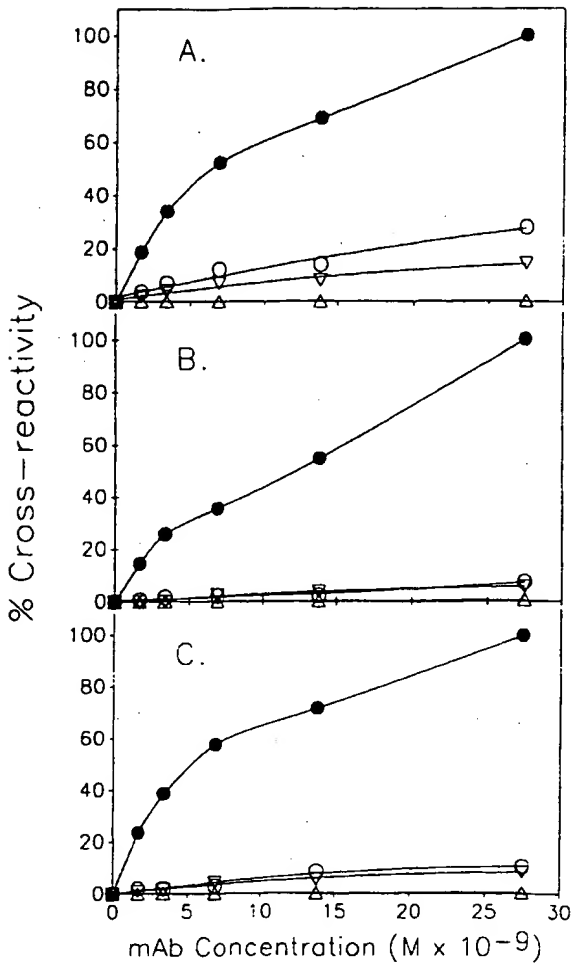

Fig. 4. Cross-reactivity of antisite 3 mAbs with EdMb and BgMb which have substitutions in and outside the site. The binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentrations to microtiter wells coated with 50 μl of SpMb (●), EdMb (▽), and BgMb (○) having the same antigen concentration (50 μg/ml) was done as shown under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to maximum bound by SpMb as 100%) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, EdMb, and DgMb.

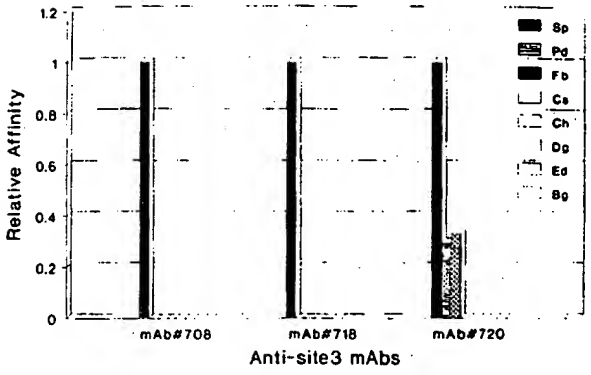

Fig. 5. Relative binding affinities of antisite 3 mAbs to myoglobin variants. The relative binding of antisite 3 mAbs to each myoglobin is calculated from mAb concentrations at 50% cross-reactivity relative to SpMb "the reference antigen" as 1.0. The relative binding affinities of mAbs 708 and 718 (to PdMb, FbMb, CsMb, ChMb, DgMb, EdMb, and BgMb) and of mAb 720 (to CsMb, ChMb, DgMb, and BgMb) could not be obtained because their maximum cross-reactivities were <50% (see Figs. 2–4).

stated that "if the substitution leads to a change in antibody binding, the mutated residue is likely to be directly involved in the structure of an epitope". And this method gives unambiguous information only when monoclonal antibodies are used as probes and the tertiary structure of the protein is known" (van Regenmortel, 1988). Based on this method of interpretation, a large number of protein antigenic sites have been described (e.g., Reichlin, 1972; Urbanski and Margoliash, 1977; Reichlin and Eng, 1978; Berzofsky *et al.*, 1982; Benjamin *et al.*, 1984; Hannum and Margoliash, 1985; Berzofsky and Berkower, 1989).

The findings mentioned above—which showed (Kazim and Atassi, 1980; Atassi and Kazim, 1980; Twining *et al.*, 1980) that substitutions outside the known antigenic sites of Mb (Atassi, 1975) and lysozyme (Atassi, 1978) influenced the antigenic reactivity of each protein with its respective antibodies—were done with polyclonal antiprotein antibodies. The specificities of these antibodies had been mapped exhaustively by a number of independent chemical, synthetic, and immunochemical approaches (Atassi, 1975, 1978). Because the polyclonal antibodies were capable of binding to a number of antigenic sites (five in Mb and three in lysozyme), the effects of substitutions on the binding properties of a given antigenic site were difficult to isolate and were estimated from the distances of the replacements from site residue (Kazim and Atassi, 1980; Twining *et al.*, 1980). A simpler system was therefore required to examine in an unequivocal manner the effect of substitutions outside an antigenic site whose boundaries are known precisely. This would also resolve whether cross-reactivities of protein mutants is a reliable approach for localization of antigenic sites. Clearly, to investigate

Table II. Nearest-Neighbour Residues to Antigenic Site 3 of Sperm Whale Mb[a]

Nearest neighbor residue	Minimum distances and closest atoms to residues in antigenic site 3						
	Ala-94	Thr-95	Lys-96	His-97	Lys-98	Ile-99	Pro-100
Lys-42					4.43 NZ-O	5.29 CG-CD_1	4.48 CE-CD
Phe-43						6.53 CE_2-CD_1	
Ala-90	5.70 O-N						
Ser-92			6.94 O-N	4.22 O-N			
His-93				4.80 O-C		5.75 O-C	
Ala-94	C-N	3.95					
Thr-95	3.94 N-C						
Lys-96				6.66 C-N			
His-97			6.66 N-C			6.79 C-N	
Ile-99				6.79 N-C			5.04 C-N
Pro-100						5.04 N-N	
Tyr-103							4.92 N-O
Leu-104						6.89 CD_2-CG_2	
Tyr-146	5.01 OH-N					6.26 OH-O	6.40 CE_1-C
Leu-149	5.85 CD_1-CB	5.01 CD_1-CG_2					
Tyr-151	5.78 CE_1-O	4.24 CE_2-CG_2					

[a] The first atom notation refers to the neighboring residue and the second atom refers to the site residue (for atom notations, see Watson, 1969). The distances of the nearest-neighbor residues from site 3 residues were calculated from the 2.0 Å coordinates of the SpMb structure (Takano, 1984).

these questions, mAbs are needed whose specificities are predetermined by the investigator and thus are precisely known. This was made possible by the discovery that small peptides (six residues or longer) can be used as immunogens in their free form (i.e., without coupling to any carrier) to generate polyclonal and mAbs of predetermined specificities (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*; Young *et al.*, 1983; for review, see Atassi, 1986). In this study a free synthetic peptide was employed as the immunizing antigen. Since a carrier was not used, no additional unknown residues (from the carrier) would contribute to binding with antibodies against the peptide. Although, clearly, any protein whose covalent and 3D structures are known could be employed as a model for such studies, we have used here SpMb, because we have already made a large number of synthetic Mb peptides and have isolated a number of myoglobins from various species. In addition, these studies will afford the opportunity to check whether it has discontinuous antigenic sites. These had been surmised to exist in Mb entirely from interpretation of cross-reaction results based on the aforementioned assumption (i.e., that replacements causing a change are part of the site).

The nearest neighbor residues of antigenic site 3 (residues 94–100), which represent the immediate (within 7.0 Å) molecular environment of the site are listed in Table II. The replacements in these nearest-neighbor residues in the Mb variants used in this study are given in Table III. The Mb variants (Table I) were selected based on the substitutions within the region 94–100. Some variants had no substitutions in that region while others have substitutions both inside and

Table III. Nearest-neighbor (Environmental) Residues to Antigenic Site 3 of Sperm Whale Mb That Undergo Substitution in Other Myoglobins and the Nature of Substitutions[a]

Antigenic site 3 environment in sperm whale Mb	Environmental residue substitutions in other myoglobins						
	PdMb	FbMb	CsMb	ChMb	DgMb	EdMb	BqMb
Lys-42				Arg			
Phe-3							
Ala-90							
Ser-92							
His-93							
Ala-94							
Thr-95							
Lys-96							
His-97							
Ile-99							
Pro-100							Val
Tyr-103						Ser	
Leu-104						Phe	
Tyr-146							
Leu-149				Phe		Phe	
Tyr-151	Phe	Phe	Phe	Phe	Phe	Phe	Phe

[a] For the distances and molecular contacts of environmental residues within 7 Å from a site residue, see Table II. Distances were calculated from the 2.0 Å coordinates of the SpMb structure (Takano, 1984).

outside the region. The Mb variants will, therefore, be divided according to these substitutions and will be discussed below.

The finding here, that PdMb, FbMb and CsMb, which have no substitutions within the region 94–100 (Table I), exhibited much lower reactivities and relative binding affinities to antisite 3 mAbs than the reference antigen, SpMb, clearly demonstrate that amino acid substitutions outside the region 94–100 can extend their influences to alter the reactivity of that region. If the region 94–100, to which the specificity of these mAbs is directed is not affected by changes elsewhere in the molecule then the cross-reactivities of each of the three myoglobins should be equal to SpMb. The amino acid replacements which are located outside antigenic site 3 and must be responsible for these changes are listed in Table IV, together with their minimum distances from the site (which ranged from 4.24–34.86 Å). Another pair of Mb variants (ChMb and DgMb), which also have no substitutions in site 3 (Table I), exhibited drastic losses in their cross-reactivities with antisite 3 mAbs (Figs. 3 and 5). These dramatic immunochemical changes obviously stemmed from effects exerted by amino acid substitutions outside the region 94–100. These substitutions are listed in Table V, together with their minimum distances from the site which varied from 4.22–33.76 Å.

Of the echidna and badger Mbs, each has a single substitution in site 3 (Table I), in addition to the substitutions elsewhere in the molecule. In this case, it will be uncertain whether to attribute the complete destruction in their cross-reactivities to the single amino acid substitution within the site or to those outside the antigenic site. It is quite likely the result of both. The minimum distances of these substitutions (which varied from 4.24–33.76 Å, from site 3) are given in Table VI.

The observed changes in the immunochemical reactivity of a given Mb variant, relative to SpMb, were most likely due to the cumulative indirect effects of amino acid substitutions outside site 3. The individual contribution of each substitution to the overall effect is difficult to determine but would be a function of the nature of the replacement and its distance from the antigenic site. Further, it should be expected that the extent of influences in a given Mb of amino acid substitutions outside the site is also dependent on the number of amino acid substitutions. This was clear in the binding properties of PdMb, FbMb, and CsMb compared to ChMb and DgMb. The latter suffered more substitutions and were accordingly much more affected. As expected, the patterns of cross-reactivities varied from one monoclonal antibody to another, probably due to some differences in the complementarity residues making up the antibody-combining sites.

Table IV. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Pacific Common Dolphin Mb, Finback Whale Mb, and California Sea Lion Mb and their closest distances (Å) from Site 3 (Residues 94–100) in Sperm Whale Mb.

A Residue no.	Residues in SpMb	Replaced by			Dist. of A to (closest residue of) site 3 in SpMb*
		In PdMb	in FbMb	In CsMb	
1	V	G	—	G	26.00 (Ala-94)
3	S	—	T	—	30.72 (Ala-94)
4	E	D	D	D	30.02 (Ala-94)
5	G	—	A	—	30.95 (Ile-99)
8	Q	..	H	—	34.86 (His-97)
12	H	N	N	N	32.17 (Ile-99)
13	V	—	I	I	26.49 (Ile-99)
15	A	G	—	G	27.29 (His-97)
21	V	L	—	L	20.42 (His-97)
22	A	—	—	V	20.17 (His-97)
27	D	—	—	E	21.96 (Ile-99)
28	I	V	—	V	16.55 (Ile-99)
31	R	—	S	—	19.16 (Pro-100)
35	S	G	G	G	16.48 (Pro-100)
45	R	K	K	K	10.91 (His-97)
51	T	-	—	S	22.84 (Ile-99)
53	A	..	—	D	24.72 (Ile-99)
54	E	D	—	—	20.72 (Ile-99)
57	A	—	—	R	23.32 (Ile-99)
66	V	N	N	K	16.85 (His-97)
74	A	—	G	G	16.03 (His-97)
83	E	D	—	—	18.08 (Ala-94)
109	E	—	D	—	13.25 (Ile-99)
116	H	—	—	Q	27.93 (Ile-99)
118	R	--	—	K	24.88 (Ile-99)
121	G	A	A	—	33.76 (Ile-99)
122	D	E	—	—	32.39 (Ile-99)
127	A	—	—	T	27.92 (Ile-99)
128	Q	—	—	H	24.13 (Pro-100)
129	G	—	A	A	24.07 (Pro-100)
132	N	—	—	K	19.97 (Pro-100)
140	K	—	—	N	17.58 (Ala-94)
147	K	—	--	R	14.67 (Ala-94)
151	**Y**	**F**	**F**	**F**	**4.24 (Thr-95)**
152	Q	H	—	—	11.49 (Ala-94)

*These values represent the shortest distance between the residues in column A to the site 94–100. Values in bold type indicate distance less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of the SpMb structure of Takano (1984).

The studies reported here and in the following papers will afford the opportunity to check whether Mb has discontinuous antigenic sites. These had been surmised to exist in Mb (East *et al.*, 1980, 1982; Berzofsky *et al.*, 1982; Benjamin *et al.*, 1984; Berzofsky and Berkower, 1989) entirely from interpretation of cross-reaction results based on the assumption that every replacement that causes a change in immunochemical reactivity is within the boundaries of the antigenic site. Amino acid residues proposed to constitute discontinuous antigenic sites and which are relevant to antigenic site 3 will be discussed here. Those residues that are relevant to the other sites are discussed in the following papers. Amino acid residues Leu-9, Val-13, Ala-53, His-116, Asp-122, and Gly-124 were proposed to be part of a discontinuous antigenic site (East *et al.*, 1980). The present work shows that the effect of these substitutions is exerted indirectly on the binding of site 3 (as well as other sites, see the following papers). From cross-reactions with two mAbs, Ala-53, His-113 and Ala-74, Ile-142 were interpreted to be in discontinuous antigenic sites (East *et al.*, 1982). The present work has revealed that these substitutions exert their effects rather indirectly on site

Table V. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Dog Mb and Chicken Mb and Their Closest Distances (Å) from Site 3 (Residues 94–100) in Sperm Whale Mb

A Residue no.	Residues in SpMb	Replaced by		Dist. of A to (Closest residue of) site 3 in SpMb[a]
		In DgMb	In ChMb	
1	V	G	G	26.0 (Ala-94)
4	E	D	D	30.02 (Ala-94)
5	G	—	Q	30.95 (Ile-99)
9	L	I	Q	30.23 (Ile-99)
12	H	N	T	32.55 (His-97)
13	V	I	I	26.49 (Ile-99)
15	A	G	G	27.29 (His-97)
19	A	T	—	27.31 (His-97)
21	V	L	I	20.43 (His-97)
26	Q	—	H	19.07 (Ile-99)
27	D	E	E	21.96 (Ile-99)
28	I	V	V	16.55 (Ile-99)
30	I	—	M	18.59 (Ile-99)
34	K	—	H	21.08 (Ile-99)
35	S	N	D	16.48 (Pro-100)
41	E	D	D	13.60 (Pro-100)
42	**K**	**—**	**R**	**4.43 (Lys-98)**
45	R	K	K	10.91 (His-97)
48	H	—	G	18.13 (His-97)
53	A	D	P	26.87 (His-97)
54	E	—	D	20.72 (Ile-99)
57	A	G	G	23.32 (Ile-99)
66	V	N	Q	16.85 (His-97)
74	A	G	—	16.03 (His-97)
75	I	—	Q	13.30 (His-97)
85	E	—	D	16.60 (Ala-94)
92	**S**	**—**	**T**	**4.22 (His-97)**
101	I	V	V	8.68 (Pro-100)
109	E	D	-	16.22 (Pro-100)
110	A	—	V	14.19 (Ile-99)
113	H	Q	K	22.04 (Ile-99)
116	H	Q	A	27.93 (Ile-99)
117	S	—	E	24.28 (Ile-99)
118	R	K	K	24.88 (Ile-99)
120	P	S	A	29.87 (Ile-99)
121	G	—	A	33.76 (Ile-99)
124	G	H	-	28.74 (Ile-99)
127	A	T	S	27.92 (Ile-99)
128	Q	E	—	24.13 (Pro-100)
129	G	A	A	24.07 (Pro-100)
132	N	L	K	19.97 (Pro-100)
140	K	N	D	20.50 (Thr-95)
142	I	—	M	8.98 (Ala-94)
144	A	—	S	11.39 (Ala-94)
149	**L**	**—**	**F**	**5.01 (Thr-95)**
151	**Y**	**F**	**F**	**4.24 (Thr-95)**

[a] These values represent the shortest distance between the residues in column A to the site 94–100. Values in bold type indicate distance is less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of SpMb structure of Takano (1984).

Table VI. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Badger Mb and Echidna Mb and Their Closest Distances (Å) from Site 3 (Residues 94–100) In Sperm Whale Mb

A Residue no.	Residues in SpMb	Replaced by		Dist. of A to (Closest residue of) site 3 in SpMb[a]
		In BgMb	In EdMb	
1	V	G	G	26.0 (Ala-94)
4	E	D	D	30.02 (Ala-94)
12	H	N	K	32.17 (Ile-99)
15	A	G	G	27.29 (His-97)
19	A	—	T	27.31 (His-97)
21	V	L	I	20.43 (His-97)
22	A	—	T	20.17 (His-97)
27	D	E	—	21.96 (Ile-99)
28	I	V	V	16.55 (Ile-99)
35	S	G	T	16.48 (Pro-100)
45	R	K	K	10.91 (His-97)
51	T	S	—	22.84 (Ile-99)
53	A	D	—	24.72 (Ile-99)
57	A	G	—	23.32 (Ile-99)
59	E	—	A	21.65 (His-97)
66	V	N	G	16.85 (His-97)
67	T	—	V	9.06 (His-97)
74	A	G	S	16.03 (His-97)
81	H	—	Q	24.07 (Ala-94)
82	H	Q	—	15.63 (Ala-94)
100	P	—	S	5.04 (Ile-99)
101	I	V	—	8.68 (Pro-100)
103	Y	—	F	4.92 (Pro-100)
109	E	D	—	16.22 (Pro-100)
112	I	A	—	19.06 (Pro-100)
113	H	Q	—	22.04 (Ile-99)
116	H	Q	Q	27.93 (Ile-99)
118	R	K	K	24.88 (Ile-99)
120	P	—	S	29.87 (Ile-99)
121	G	—	A	33.76 (Ile-99)
122	D	N	—	32.39 (Ile-99)
124	G	A	—	28.82 (Ile-99)
126	D	E	—	31.41 (Ile-99)
129	G	—	A	24.07 (Pro-100)
132	N	K	G	19.97 (Pro-100)
140	K	N	N	17.58 (Ala-94)
142	I	—	I	8.98 (Ala-94)
149	L	—	F	**5.01 (Thr-95)**
151	Y	F	F	**4.24 (Thr-95)**

[a] These values represent the shortest distance between the residue in column A to the site 94–100. Values in bold type indicate distance is less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of SpMb structure of Takano (1984).

3 (as well as on other sites as seen in the following papers). Similarly, Glu-4, Glu-83, Ala-144, and Lys-140, which had been proposed to reside within discontinuous sites (Berzofsky *et al.*, 1982), their effects were found here to be in nature indirectly exerted on the reactivity of site 3 (as well as other sites as shown in the following papers). The mAbs employed in those studies (East *et al.*, 1982; Berzofsky *et al.*, 1982) were generated by using whole Mb as the immunogen and, therefore, their site specificity was not known but was totally surmised from cross-reaction studies with Mb variants, each of which had many substitutions, such as the Mbs used in the present work. The results reported here and in the following papers with peptide-elicited mAbs clearly show that such interpretations are not correct.

In conclusion, the results with antisite 3 mAbs have shown that amino acid substitutions outside this

region, which by design were not part of the recognition site of antisite 3 mAbs can exert destructive effects, on the binding of that region. Clearly, therefore, it is incorrect to assume that every mutation affecting the binding of a protein to mAbs should be part of an antigenic site.

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β-Endorphin omissi n analogs: Dissociation of immunoreactivity from other biological activities

(peptide synthesis/radioimmunoassay/analgesia/ileal opiate activity/opiate receptor-binding activity)

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ABSTRACT An analog of human β-endorphin with omission of four residues at positions 11, 14, 20, and 22 has been synthesized. This analog and other synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 have been assayed for analgesic potency, ileal opiate activity, opiate receptor-binding activity, and immunoreactivity. Results show that deletion of a single amino acid of the β-endorphin molecule outside of the enkephalin segment to give des-Gln[11]-, des-Thr[12]-, des-Pro[13]-, des-Leu[14]-, des-Val[15]-, des-Asn[20]-, or des-Ile[22]-β-endorphin markedly reduced or abolished the immunoreactivity yet gave substantial retention of opiate potencies. Deletion of a single amino acid of β-endorphin within the enkephalin segment (des-Gly[2]- or des-Met[5]-β-endorphin) did not markedly affect the immunoactivity; however, the opiate activities were abolished or markedly reduced. The data indicate a clear dissociation of immunoactivity from analgesic, ileal-opiate, and opiate receptor-binding activities.

β-Endorphin (β-EP) (ref. 1; see Fig. 1) is a naturally occurring opioid peptide with potent opiate analgesic activity after intracerebral (2) or intravenous injections (3, 4). Studies on structure–activity relationships indicate that the entire β-EP molecule is necessary for full analgesic potency (5). In addition, omission of a single amino acid residue at position 14 or 20 abolishes immunoreactivity yet gives retention of opiate potency (6). We present herein biological activities of synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 as well as a synthetic analog with omission of four residues at positions 11, 14, 20, and 22. The analogs were assayed for analgesic activity by the tail-flick test, ileal opiate activity by the guinea pig ileum method, opiate receptor-binding activity by displacement of [^{3}H]-β-EP binding to membrane of rat brain, and immunoreactivity by radioimmunoassay. Results show a clear dissociation of immunoreactivity from other biological activities.

MATERIALS AND METHODS

Synthesis of single-deletion analogs of β_c-EP has been described (6, 7). Des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP was synthesized by the solid-phase method (8). It was performed with Boc(Bzl)Glu brominated polymer (0.34 mmol/g) (4) on a Beckman model 990 peptide synthesizer. A fully automated symmetrical anhydride program (5) was used except for the Asn residue, which was incorporated by procedures described for the synthesis of β_h-EP (4). The following amino acid residues in the β_h-EP sequence were omitted in the synthesis: Gln[11], Leu[14], Asn[20], and Ile[22]. From 295 mg (100 μmol) starting resin ther was obtained, after removal of the last Boc group, 768 mg of protected peptide resin corresponding to des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP. Cleavage and deprotection in HF (6), gel filtration on Sephadex G-10 (0.5 M acetic acid), and chromatography on carboxymethylcellulose were performed as described (4). From additivity rules for the hydrophobicities of amino acid residues in β-EP (7) it could be predicted that partition chromatography on Sephadex G-50 in the same solvent system used for β_h-EP (4) would give an R_F of 0.27 based on the reported R_F of 0.40 for β_h-EP. The experimental value of R_F was found to be 0.26. The overall yield of des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP based on 50 μmol of starting resin was 39.0 mg (26%). The product (50-μg samples) was homogeneous on thin-layer chromatography on silica gel in 1-butanol/pyridine/acetic acid/H_2O (5:5:1:4, vol/vol), R_F 0.40 (ninhydrin and chlorine–tolidine detection), and in paper electrophoresis on Whatman 3 MM at pH 3.7 (R_F 0.58 relative to Lys) and pH 6.7 (R_F 0.45 relative to Lys) at 400 V (5 hr, ninhydrin detection). Amino acid analysis of a 24-hr HCl hydrolysate gave (theoretical values in parentheses): Lys, 4.91 (5); Asp, 1.06 (1); Thr, 3.12 (3); Ser, 1.92 (2); Glu, 2.10 (2); Pro, 0.96 (1); Gly, 2.97 (3); Ala, 2.12 (2); Val, 1.02 (1); Met, 0.98 (1); Ile, 1.00 (1); Leu, 1.07 (1); Tyr, 1.96 (2); Phe, 1.96 (2). Amino acid analysis of an enzymic digest (trypsin and chymotrypsin followed by leucine aminopeptidase) gave: Lys, 4.90 (5); Thr + Ser + Asn, 6.30 (6); Glu, 1.96 (2); Pro, 0.91 (1); Gly, 2.84 (3); Ala, 2.14 (2); Val, 1.12 (1); Met, 0.98 (1); Ile, 1.06 (1); Leu, 1.10 (1); Tyr, 1.90 (2); Phe, 1.84 (2).

```
                    5                  10
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-

                   15                  20
Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                   25                  31
Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
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FIG. 1. Amino acid sequence of β_h-EP (β_c-EP: His-27, Gln-31). Residues 1–5 correspond to [Met]enkephalin.

Opiate activities were assessed both *in vitro* and *in vivo*. The ileal opiate activity *in vitro* was measured by the inhibition of electrically stimulated contraction of guinea pig ileum preparation (10), and the piate receptor-binding assay was performed according to the procedure recently described (11, 12), using [^{3}H-Tyr[27]]-β_h-EP (13) as the primary ligand and synthetic

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Abbreviations: β-EP, β-endorphin (subscripts h and c indicate β-EP from human and camel pituitaries); IC_{50}, 50% inhibitory concentration.

Table 1. Ileal opiate activities of omission analogs of β-EP

Synthetic peptide	IC_{50},* nM	Relative potency
β_c-EP	91	100
Des-Gly^2-β_c-EP	9100	1
Des-Leu^{14}-β-EP	65	140
β_c-EP	58	100
Des-Thr^6-β_c-EP	60	97
Des-Ser^{10}-β_c-EP	49	118
Des-Thr^{12}-β_c-EP	50	116
β_c-EP	132	100
Des-Met^5-β_c-EP	2000	7
Des-Val^{15}-β_c-EP	107	123
Des-Ile^{22}-β_c-EP	67	197
β_h-EP	22	100
β_c-EP	22	100
Des-Gln^{11}-β_c-EP	22	100
Des-Pro^{13}-β_c-EP	20	110
Des-Asn^{20}-β_c-EP	20	110
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	16	138

* Guinea pig ileum assay; IC_{50} is the concentration that gives 50% inhibition of contraction.

β_c-EP (14) or β_h-EP (4) as standard competing ligand. The analgesic activity *in vivo* was assessed in mice by the tail-flick method (15) as described (5). Radioimmunoassay was carried out by the procedure described (16, 17).

RESULTS

The opiate activities *in vitro* of various omission analogs as assayed by the guinea pig ileum preparation are summarized in Table 1. Deletion of Gly^2 or Met^5 in the [Met]enkephalin segment of β-EP causes a marked decrease of opiate potency. On the other hand, deletion of a single amino acid residue outside the [Met]enkephalin segment does not alter the opiate potency and in some cases even enhances it. For example, des-Ile^{22}- and des-Leu^{14}-β_c-EP are 1.4 and 1.97 times as potent as the intact peptide, respectively. Omission of four residues in positions 11, 14, 20, and 22 increases the potency to 138% compared with β_h-EP.

Table 2. Analgesic potencies of omission analogs of β-EP

Synthetic peptide	AD_{50}*, nmol/mouse	Relative potency
β_c-EP	0.026 (0.020–0.032)	100
Des-Gly^2-β_c-EP	>25	<0.1
Des-Gln^{11}-β_c-EP	0.033 (0.021–0.048)	79
Des-Pro^{13}-β_c-EP	0.113 (0.089–0.149)	23
Des-Asn^{20}-β_c-EP	0.057 (0.042–0.075)	46
β_c-EP	0.043 (0.035–0.075)	100
Des-Met^5-β_c-EP	0.219 (0.099–0.487)	20
Des-Thr^6-β_c-EP	0.059 (0.048–0.077)	73
Des-Ser^{10}-β_c-EP	0.047 (0.027–0.092)	92
Des-Thr^{12}-β_c-EP	0.045 (0.024–0.095)	96
Des-Leu^{14}-β_c-EP	0.057 (0.033–0.093)	75
Des-Val^{15}-β_c-EP	0.179 (0.131–0.241)	24
Des-Ile^{22}-β_c-EP	0.076 (0.045–0.117)	57
β_h-EP	0.064 (0.026–0.17)	100
Des-Gln^{11},Leu^{14},Asn^{20}, Ile^{22}-β_h-EP	0.99 (0.45–2.19)	7

* Median antinociceptive dose (95% confidence limit).

Table 3. Opiate receptor-binding activities of omission analogs of β-EP

Synthetic peptide	IC_{50}, pM	Relative potency
β_c-EP	250	100
Des-Gly^2-β_c-EP	50,000	0.5
Des-Met^5-β_c-EP	12,000	2
Des-Thr^6-β_c-EP	550	45
Des-Ser^{10}-β_c-EP	280	90
Des-Gln^{11}-β_c-EP	210	120
Des-Thr^{12}-β_c-EP	270	93
Des-Pro^{13}-β_c-EP	860	29
Des-Leu^{14}-β_c-EP	430	58
Des-Val^{15}-β_c-EP	390	64
Des-Asn^{20}-β_c-EP	530	47
Des-Ile^{22}-β_c-EP	180	139
β_h-EP	560	100
β_c-EP	250	224*
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	610	92*, 41†

* Relative to β_h-EP.
† Relative to β_c-EP.

Table 2 presents the analgesic potencies *in vivo* of the synthetic analogs. Des-Gly^2-β_c-EP is less than 0.1% as potent as β_c-EP, and des-Met^5-β_c-EP exhibits only 20% potency relative to that of β_c-EP. The majority of analogs with deletion of a single amino acid residue outside the [Met]enkephalin segment retain substantial analgesic potency. However, deletion of four residues (positions 11, 14, 20, and 22) drops potency to 7% of that of the intact molecule.

As in the ileal opiate activity assay, the deletion of a single amino acid residue at position 2 or 5 markedly reduced the potency in the opiate receptor assay using membranes of rat brain. Deletion of a single amino acid residue outside the enkephalin segment, however, did not markedly alter the opiate receptor-binding potency (Table 3). Even deletion of four residues simultaneously had no drastic effects.

Table 4 summarizes the immunoreactivity of omission analogs by the β_h-EP radioimmunoassay system. Deletion of a single amino acid residue at position 11, 12, 13, 14, 15, or 20 abolished or markedly reduced the abilities of these peptides

Table 4. Immunoactivity of omission analogs of β-EP

Synthetic peptide	IC_{50},* pM	Relative activity
β_c-EP	72	100
Des-Gln^{11}-β_c-EP	800	9
Des-Pro^{13}-β_c-EP	1,029	7
Des-Leu^{14}-β_c-EP	>10,000	<1
Des-Val^{15}-β_c-EP	>10,000	<1
Des-Asn^{20}-β_c-EP	>10,000	<1
Des-Ile^{22}-β_c-EP	248	29
β_c-EP	56	100
Des-Gly^2-β_c-EP	68	82
β_h-EP	51	100
β_c-EP	51	100
Des-Met^6-β_c-EP	67	76
Des-Thr^6-β_c-EP	48	106
Des-Ser^{10}-β_c-EP	70	73
Des-Thr^{12}-β_c-EP	1,300	3
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	>10,000	<1

* Radioimmunoassay.

to bind to the antibodies of β-EP. On the other hand, omission of Gly[2] or Met[5] in the [Met]enkephalin segment of β_c-EP as well as Thr[6] or Ser[10] resulted in retention of high immunoreactive potency.

DISCUSSION

Previous studies indicated the importance of the Tyr[1], Phe[4], and Met[5] residues for the production of opiate analgesic activity (18–20). In this study, we found that deletion of Gly[2] or Met[5] in the [Met]enkephalin segment of β-EP drastically lowers opiate analgesic, ileal opiate, and receptor-binding potency, whereas nearly full immunoreactivity is retained. Of these two residues, Gly[2] appears to be more important for the production of opiate activities. Omission of a single residue outside of this segment does not cause considerable loss of biological activities, but immunoreactivity is markedly affected. Omission of a single amino acid at position 14, 15, or 20 abolishes immunoreactivity yet retains significant amounts of other biological activities. This indicates that the active sites in the β-EP molecule for binding to the β-EP antibodies resides in positions 11 to 22. Thus we have discovered an instance in which deletion of a single amino acid residue in a biologically active peptide abolishes immunoreactivity.

Des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP has virtually no immunoreactivity and exhibits somewhat higher ileal opiate and significant receptor-binding activity in comparison to β_h-EP. Analgesic potency of this analog is only 7% when compared with the activity for the intact molecule. In an earlier report (16), a lack of correlation between immunoreactivity and opiate activity as assayed by the guinea pig ileum preparation has been noted.

When the relative ileal opiate activities for des-Gln[11]-, des-Leu[14]-, des-Asn[20]-, and des-Ile[22]-β_c-EP are averaged, a value of 137 is obtained. It is interesting that the observed opiate activity of des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP is 138. On the other hand, the similarly calculated values for the other activities of des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP (receptor-binding activity, 124; analgesic potency, 64, and immunoreactivity, 10) diverge increasingly from the experimental data in the order given. These results illustrate the insensitivity of the ileal assay to such structural alterations, while the other assays show sensitivity in the order immunoreactivity > analgesic activity > receptor-binding activity.

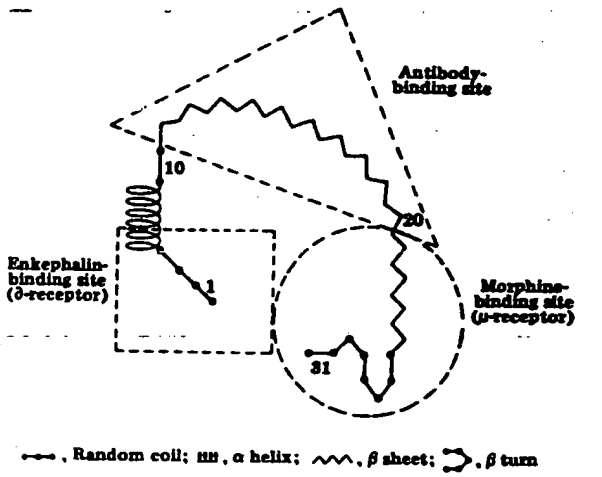

FIG. 2. Proposed binding sites in the primary structure of β-EP. Predicted secondary structure of β_h-EP was taken from ref. 23.

The data, summarized in Table 5, clearly show the dissociation of immunoreactivity from analgesic, ileal opiate, and receptor-binding activities. Moreover, there is a fair correlation between analgesic potency and receptor-binding activity if des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP is excluded. This omission analog possesses significant receptor-binding activity and low analgesic potency. The lack of correlation between opiate receptor-binding activity and analgesic potency has recently been observed with synthetic analogs with extension at the COOH terminus (21). These data emphasize again the importance of not relying on a single assay procedure for the characterization of biologically active peptides.

There are at least two receptors for opioid peptides in the brain (22): the μ receptors for morphine and the ∂ receptors for the enkephalins. The data presented herein, together with the recent findings that β_c-EP-(6–31) and β_c-EP-(20–31) segments inhibit morphine-induced analgesia (unpublished), suggest the presence of three binding sites in the β-EP molecule as shown in Fig. 2. The first site resides in the [Met]enkephalin segment [enkephalin-binding site ("∂-receptor")] and the second consists

Table 5. Relative biological activities of omission analogs of β-EP

Synthetic peptide	Analgesic potency	Opiate activity	Receptor-binding activity	Immuno-reactivity
β_c-EP	100	100	100	100
Des-Gly[2]-β_c-EP	<0.01	1	0.5	82
Des-Met[5]-β_c-EP	20	7	2	76
Des-Thr[6]-β_c-EP	73	97	45	106
Des-Ser[10]-β_c-EP	92	118	90	73
Des-Gln[11]-β_c-EP	79	100	120	9
Des-Thr[12]-β_c-EP	96	116	93	3
Des-Pro[13]-β_c-EP	23	110	29	7
Des-Leu[14]-β_c-EP	75	140	58	<1
Des-Val[15]-β_c-EP	24	123	64	<1
Des-Asn[20]-β_c-EP	46	110	47	<1
Des-Ile[22]-β_c-EP	57	197	139	29
β_h-EP	100	100	100	100
β_c-EP	100	100	224*	100
Des-Gln[11],Leu[14],Asn[20],Ile[22]-β_h-EP	7	138	92*, 41†	<1

* Relative to β_h-EP.
† Relative to β_c-EP.

of the COOH-terminal segment [β-EP-(21–31)] [m rphine-binding sit ("μ-receptor")]. The middle segment [β-EP-(11–20)] is the antibody-binding site. Studies on th *in vivo* and *in vitro* biological profiles of synthetic β-EP analogs may possibly clarify the role of these binding sites.

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Anti-PDI

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Rabbit Anti-Protein Disulfide Isomerase Polyclonal Antibody
Product #: SPA-890

Immunogen
Purified bovine liver Protein Disulfide Isomerase (PDI).

Specificity
This antibody identifies purified bovine PDI protein and will detect an ~58 kDa protein, corresponding to the apparent molecular mass of PDI on SDS-PAGE immunoblots, in samples from human, mouse, rat, monkey, hamster, guinea pig, bovine, sheep, pig, dog, and *Xenopus* origins. This antibody identifies purified bovine PDI protein.

Species Reactivity
human, mouse, rat, monkey, hamster, guinea pig, bovine, sheep, pig, dog, *Xenopus*

Applications	Certified*	Cited
Western Blot (Colorimetric) (4,5,8,9,11)	1:4,000	
Western Blot (ECL) (12,13)		1:5,000
Immunoprecipitation (11,16)		1:100
Immunocytochemistry (6,10,11,14,15)		Yes

*These working dilutions are provided as suggestions only. Further dilutions may be possible. Each user should determine the optimal conditions for their own particular experiment.

Positive Controls
Purified Bovine PDI Protein, Product#: SPP-890
HeLa Cell Lysate, Product#: LYC-HL100

Scientific Background
The mammalian protein disulphide-isomerase (PDI) family encompasses several highly divergent proteins which are involved in the processing and maturation of secretory proteins in the ER by catalyzing the rearrangement of disulphide bonds (2,7). PDI, which is an abundant protein of the ER (>400uM), has a carboxy-terminal retention signal sequence, KDEL, similar to that of BiP and Grp94 (1). The PDI proteins are characterized by the presence of one or more domains of ~95-110 amino acids related to the cytoplasmic protein thioredoxin (2). All but the PDI-D subfamily are composed entirely of repeats of such domains, with at least one domain containing and one domain lacking a redox-active-Cys-Xaa-Xaa-Cys-tetrapeptide (2). In addition to their roles as redox catalysts and isomerases, PDI proteins have other functions such as peptide binding, cell adhesion and perhaps chaperone activities (2). Platelet surface thiols and disulphides play an important role in platelet responses (3). Catalytically active PDI is found on platelet surfaces where it has been demonstrated to mediate platelet aggregation and secretion possible by reducing disulfide bonds thus leading to exposure of fibrinogen receptors in platelets (3).

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Rev: 03/11/03

CERTIFICATE OF ANALYSIS

Anti-PDI

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Lot #: B302407
Format: Whole rabbit serum

Host: Rabbit **Antibody Type:** Polyclonal
Certification: A 1:4,000 dilution of SPA-890 was sufficient for detection of PDI in 50ng of purified bovine PDI protein (product# SPP-890) by colorimetric immunoblot analysis using Stressgen's Goat anti-Rabbit IgG:AP (product# SAB-301) as the secondary antibody.

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MATERIAL SAFETY DATA SHEET

Secti n 1 - Product Identification and Us

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This product is sold only for research use by qualified laboratory personnel, and is not to be used as a drug, medical device, food additive, cosmetic, nor household chemical. It is not to be used in diagnostic, therapeutic, consumer, agricultural, nor pesticidal applications.

Manufacturer's Name:	Stressgen Biotechnologies Corp.
Street Address:	120-4243 Glanford Avenue
City, Prov. Postal Code:	Victoria, B.C., CANADA V8Z 4B9
Fax:	(250) 744-2877
EMERGENCY PHONE:	(250) 744-2811

Section 2 - Hazardous Ingredients

NOT AVAILABLE. We are not aware of any hazards associated with this product or its ingredients, but the chemical, physical, and toxicological properties of this product have not been investigated thoroughly. Observe normal laboratory precautions.

Section 3 - Physical Data

This product consists of whole rabbit serum shipped on gel packs (dry ice overseas). The physical properties of this product have not been investigated thoroughly.

Section 4 - Fire and Explosion Hazard

NOT APPLICABLE

Section 5 - Reactivity Data

NOT APPLICABLE

Section 6 - Toxicological Properties

May be harmful by inhalation, ingestion, or skin absorption. The toxicological properties of this product have not been investigated thoroughly. Exercise due caution.

Section 7 - Preventative Measures

Wear chemical safety goggles and compatible chemical-resistant gloves. Avoid inhalation, contact with eyes, skin or clothing.

*****MULTIPLE COMPONENT SPILL OR LEAK PROCEDURES*****

- Wear protective equipment.
- Absorb on sand or vermiculite and place in closed containers for disposal.
- Dispose or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber. Observe all federal, state and local environmental regulations.

Section 8 - First Aid Measures

- If swallowed, wash out mouth with water, provided person is conscious. Call a physician.
- In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes. If a rash or other irritation develops, call a physician.
- If inhaled, remove to fresh air. If breathing becomes difficult, call a physician.
- In case of eye contact, flush with copious amounts of water for at least 15 minutes while separating the eyelids with fingers. Call a physician.

Section 9 - Preparation

Prepared by:	Phone#:	Created:	Modified:
SBC	(250) 744-2811	03/11/03	03/11/03

PDI Antibody - Rabbit Polyclonal

Catalog Number	Format	Tested Applications	Size	Price	Quantity to Add to Cart
SPA-890D	Antiserum	WB, IP, ICC	50ul	$144.00	1 Add
SPA-890F	Antiserum	WB, IP, ICC	200ul	$319.00	1 Add

This antibody detects an ~58 kDa band corresponding to the apparent molecular mass of Protein Disulfide Isomerase (PDI) on SDS-PAGE immunoblots.

Tech Spec Download

Immunogen Bovine PDI

Antigen The predicted molecular weight of the antigen, 57,265 (precursor protein) Daltons, is calculated from the sequence reported in M17596 in the GenBank database (unless stated otherwise). This information is provided as a guide only; empirical results may differ.

Reactivity human, mouse, rat, bovine, canine, guinea pig, hamster, monkey, pig, sheep, Xenopus

Positive Controls HeLa Cell Lysate, LYC-HL100

Recommended Secondary Antibodies Anti-Rabbit Total IgG, Conjugated to HRP, AP, Biotin, or FITC

See our selection of secondary antibodies.

Search for Citations **of this product**

List the Product Groups **this product belongs to.**

Add Additional Products

Attachment 8 of 11
In USSN: 09/847,809
PF-0358-2 DIV

1: M17596. Bovine protein di...[gi:163496]

Links

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  TITLE     Sequence of membrane-associated thyroid hormone binding protein
            from bovine liver: its identity with protein disulphide isomerase
  JOURNAL   Biochem. Biophys. Res. Commun. 146 (3), 1485-1492 (1987)
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   PUBMED   3619939
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  AUTHORS   Yamauchi,K., Yamamoto,T., Hayashi,H., Koya,S., Takikawa,H.,
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  TITLE     Sequence of membrane-associated thyroid hormone binding protein
            from bovine liver: its identity with protein disulphide isomerase
  JOURNAL   Biochem. Biophys. Res. Commun. 146 (3), 1485-1492 (1987)
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Retrieval | BLAST2 | FASTA | ClustalW | GCG Assembly | Phrap | Translation

 SeqServer Version 4.6 Jan 2002

- [ ] 1601793CD1
- [ ] GI:163497

CLUSTAL W (1.7) Multiple Sequence Alignments

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Aligning...
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Submit sequences to: BLAST2 ▼ Submit Reset

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Printed in U.S.A.

Reticulocalbin, a Novel Endoplasmic Reticulum Resident Ca^{2+}-binding Protein with Multiple EF-hand Motifs and a Carboxyl-terminal HDEL Sequence*

(Received for publication, June 23, 1992)

Masayuki Ozawa‡ and Takashi Muramatsu

From the Department of Biochemistry, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890, Japan

A novel Ca^{2+}-binding protein, tentatively designated r ticulocalbin, has been identified and characterized. Reticulocalbin is a luminal protein of the endoplasmic reticulum with an M_r of 44,000 as revealed by biochemical analysis and immunofluorescence staining. The cDNA of reticulocalbin encodes a protein of 325 amino acids with an amino-terminal signal sequence of 20 amino acids. The protein has six repeats of a domain containing the high affinity Ca^{2+}-binding motif, the EF-hand. Although oxygen-containing amino acids imp rtant for the positioning of Ca^{2+} are conserved in all six domains, the conserved glycine residues in the central portion of the EF-hand motif are absent in three of them. Calcium blots showed that recombinant reticulocalbin expressed in bacterial cells binds Ca^{2+}. The protein has the sequence His-Asp-Glu-Leu (HDEL) at its carboxyl terminus. This is similar to the Lys-Asp-Glu-Leu sequence, which serves as a signal to retain the resident proteins in the endoplasmic reticulum of animal cells. A mutant protein lacking the HDEL sequence produced by *in vitro* mutagenesis has been shown to be secreted into medium in transient expression assays.

Ca^{2+} is involved in the regulation of various cellular activities such as contraction, secretion, and mitogenesis. These events are largely mediated by a family of homologous Ca^{2+}-binding proteins including calmodulin, troponin C, calbindins, and S-100 proteins. These proteins exhibit a common structural motif, the EF-hand (Kretsinger, 1980), which is present in multiple copies (calmodulin and troponin C have four and S-100 proteins have two copies per molecule) and binds Ca^{2+} selectively, with high affinity. Each of these consists of a loop of 12 amino acids that is flanked by two α-helices (Strynadka and James, 1989; Heizman and Hunziker, 1991). Upon Ca^{2+} binding, these proteins undergo a conformational change and consequently interact with their target proteins.

The lumen of the endoplasmic reticulum (ER)[1] (reticuloplasm) contains a number of soluble proteins including immunoglobulin heavy chain-binding protein (GRP78) (Munro and Pelham, 1986; Bole *et al.*, 1986), protein disulfide-isomerase (Freedman, 1989; Edman *et al.*, 1985), and GRP94 (endoplasmin, ERp99) (Mazzarella and Green, 1987; Sorger and Pelham, 1987). Many of these proteins are involved in the initial steps of the maturation of newly synthesized secretory proteins such as folding of nascent polypeptide chains and formation of the correct disulfide bonds. Retention of these resident proteins in the ER is dependent on a carboxyl-terminal signal, which in animal cells is usually Lys-Asp-Glu-Leu (KDEL). The KDEL sequence is recognized by a membrane-bound receptor that continually retrieves the proteins from a later compartment (*cis*-Golgi cisternae) of the secretory pathway and returns them to the ER (Pelham, 1989, 1990). Although the above-mentioned proteins (immunoglobulin heavy chain-binding protein, protein disulfide-isomerase, and GRP94) reportedly bind Ca^{2+} (Macer and Koch, 1988), none of them have the EF-hand motifs.

Calreticulin, a well-characterized Ca^{2+}-binding protein of the ER and the sarcoplasmic reticulum, binds Ca^{2+} with high affinity, but does not have an EF-hand motif (Smith and Koch, 1989; Fliegel *et al.*, 1989). Calreticulin may be a nonmuscle functional analogue of calsequestrin, a major Ca^{2+}-binding (storage) protein of the skeletal muscle sarcoplasmic reticulum membrane (Milner *et al.*, 1991). So far, there have been no reports of ER resident proteins having EF-hand motifs.

We previously isolated several independent cDNA clones from λgt11 libraries of mouse teratocarcinoma OTT6050 (Ozawa *et al.*, 1988; Furukawa *et al.*, 1990). These clones were isolated by screening the libraries with antibodies against *Dolichos biflorus* agglutinin-binding glycoproteins. The lectin *D. biflorus* agglutinin is known to bind specifically with the nonreducing terminal *N*-acetylgalactosamine of carbohydrate chains (Etzler, 1972). Although the majority of the clones showed developmentally regulated expression, others did not, suggesting that the latter are products of housekeeping genes. Sequencing a group of cDNA clones that belong to the latter category and characterizing the protein encoded by these clones revealed that the protein is an ER resident Ca^{2+}-binding protein with multiple EF-hand motifs for which we propose the nomenclature reticulocalbin.

MATERIALS AND METHODS

cDNA Cloning—cDNA clones O1, O9, and O32 were isolated from a λgt11 cDNA library of mouse teratocarcinoma OTT6050 (Ozawa *et al.*, 1988) by screening with antibodies against *D. biflorus* agglutinin-binding glycoproteins as previously described (Ozawa *et al.*, 1988). Clones M10 and M22 were obtained from a primer extension cDNA library in λgt10 and subcloned into pUC18 and Bluescript KS(+)

* This work was supported by grants from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D13003.

‡ To whom correspondence should be addressed. Tel.: 81-992-64-2211; Fax: 81-992-64-5618.

[1] The abbreviations used are: ER, endoplasmic reticulum; bp, base pair(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ConA, concanavalin A; PAGE, polyacrylamide gel electrophoresis; MBP, maltose-binding protein.

(Stratagene) vectors, and the DNA was sequenced from both strands as described (Ozawa *et al.*, 1988).

Construction of Expression Vectors—To express reticulocalbin in animal cells, the cDNA was cloned into a mammalian expression vector, pCAGGS, which contains an enhancer derived from cytomegalovirus and the β-actin promotor (Niwa *et al.*, 1991). The Bluescript KS(+) vector containing the 1052-bp 5′-fragment of reticulocalbin cDNA in the *Eco*RI-*Pst*I site was restricted with *Sma*I and *Hinc*II. The fragment was isolated and cloned into pCAGGS, which had been digested with *Eco*RI and filled in with T4 DNA polymerase. The orientation of the cDNA in the vector was confirmed by restriction enzyme digestion. We constructed an expression vector encoding a mutant reticulocalbin lacking the carboxyl-terminal HDEL peptide by polymerase chain reaction (PCR). Oligonucleotides GACATCGACAAGAACGG and CCTGCAGTCAATTTTTGGTCAGGTCTTCC were synthesized and used as primers. The former corresponds to the sequence of the cDNA from positions 656 to 672. The latter contains a complementary sequence of the cDNA at positions 979–997 and the TGA termination codon as well as a *Pst*I recognition sequence at the 5′-end. The template was the 1052-bp reticulocalbin cDNA in the Bluescript vector, which was linearized by digestion with *Pvu*II. PCR was performed according to the manufacturer's instructions using the GeneAmp PCR reagent kit (Perkin-Elmer Cetus Instruments). The reaction mixture was subjected to 25 cycles of denaturation (93 °C, 1 min), annealing (50 °C, 2 min), and extension (72 °C, 3 min). The PCR product was purified on agarose gel, digested with *Pst*I, and cloned into the *Sma*I-*Pst*I site of Bluescript KS(+). After confirming the sequence of the 3′-*Bam*HI-*Pst*I fragment, the 5′-region was replaced with the authentic fragment and cloned into the pCAGGS vector as described above.

To express recombinant reticulocalbin in *Escherichia coli*, the cDNA encoding the mature protein was cloned into the maltose-binding protein fusion vector (pMAL-c) (New England BioLabs, Inc.) as follows. The cDNA region coding for the signal sequence was eliminated by PCR. We synthesized oligonucleotide CAGCTGCGGGCCAAGCCCACG for use as a primer. It corresponds to the nucleotide sequence encoding the first 6 amino acid residues of the mature protein with the 5′-extension of CAG, which, together with CTG, constitutes a recognition sequence for *Pvu*II. The second primer was oligonucleotide CAGGAAACAGCTATGAC, which was purchased commercially as a primer for dideoxy sequencing (the reverse primer; Takara, Kyoto, Japan). The template, the PCR program, and other conditions were the same as described above. The PCR product was cloned into the *Sma*I-*Pst*I site of Bluescript KS(+) after the fill-in reaction and *Pst*I digestion, and the sequence was confirmed. A 959-bp *Pvu*II-*Pst*I fragment that encodes the entire mature protein was cloned into the *Stu*I-*Pst*I site of the pMAL-c vector. The plasmid DNA was introduced into TB1 cells.

Cells—The mouse parietal endodermal cell line PYS-2, L cells, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 10% CO_2 atmosphere at 37 °C. Genes were transfected by the DEAE-dextran method. Ten micrograms of DNA was mixed with DEAE-dextran (400 μg/ml) and added to COS cells (3×10^5) that were plated 1 day in advance. After 4 h, the cells were washed and incubated with 100 μM chloroquine for 3 h. Cells were cultured for 2 days and analyzed.

Biochemical Analysis—Immunoblot analysis was carried out as described before (Ozawa *et al.*, 1989). Calcium blotting was performed as previously described (Maruyama *et al.*, 1984). For immunoprecipitation, 5×10^5 cells were preincubated for 30 min in Dulbecco's modified Eagle's medium without methionine and 10% dialyzed fetal calf serum and subsequently labeled with [^{35}S]methionine (1000 Ci/mmol; Du Pont-New England Nuclear) at 100 μCi/ml for 30 min. Cells were washed with normal Dulbecco's modified Eagle's medium with 10% fetal calf serum and 2 mM cold methionine and incubated in this medium for 2 h. Cells were washed with PBS and lyzed with PBS containing 1% Nonidet P-40, 1 mM $CaCl_2$, and phenylmethylsulfonyl fluoride. After centrifugation, cell lysates or media were incubated with anti-reticulocalbin antibodies, and immunocomplexes were collected by protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) as previously described (Ozawa *et al.*, 1989). Lectin-agarose fractionation proceeded as follows. Cell lysates were applied to columns (0.4-ml bed volume) containing concanavalin A (ConA)-Sepharose (Pharmacia) or *Ricinus communis* agglutinin-agarose (EY Laboratories) equilibrated with PBS containing 0.1% Nonidet P-40, 1 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride. After washing with 10 ml of this buffer, materials bound to the resin were eluted with the same buffer containing 0.2 M α-methylmannoside (for ConA-Sepharose) or 0.1 M lactose (*R. communis* agglutinin-agarose). The bound and unbound materials were analyzed by SDS-PAGE and immunoblot analysis. For subcellular fractionation, cells were homogenized in 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose. The suspension was centrifuged sequentially at 900 × *g* for 10 min, 5000 × *g* for 10 min, and 100,000 × *g* for 60 min. The precipitates from each centrifugation were designated nuclear, mitochondrial, and microsomal fractions, respectively. The supernatant after the third centrifugation was retained as the soluble fraction. The microsome fraction was further solubilized with Triton X-100 (1%) or sonicated, and the supernatants after centrifugation at 140,000 × *g* for 1 h were analyzed. Phase separation of reticulocalbin in Triton X-114 was performed as described (Bordier, 1981).

Purification of Recombinant Reticulocalbin—Bacterial cells containing the fusion plasmid were cultured, and the fusion protein was induced and collected according to the manufacturer's instructions (New England BioLabs, Inc.). The cells were disrupted by sonication in PBS containing 1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride and then centrifuged. The supernatant was applied to a column of amylose resin and washed with 5–10 column volumes of the following buffers: PBS containing 0.1% Tween 20, PBS containing 0.5 M NaCl and 0.1% Tween 20, and PBS. The fusion protein was eluted with PBS containing 10 mM maltose.

Antibodies—Antibodies were raised against *D. biflorus* agglutinin-binding glycoproteins from teratocarcinoma OTT6050 as described (Ozawa *et al.*, 1982). Monospecific antibodies against reticulocalbin were prepared as follows. The recombinant reticulocalbin of the MBP fusion described above was electrophoresed to remove any contaminating bacterial proteins. The corresponding band was excised from the gels after Coomassie Blue staining and used for immunization in rabbits. The antibodies were affinity-purified by coupling MBP or the fusion protein (1 mg each) to 1 ml of CNBr-activated Sepharose. Antibodies bound to the resin were eluted with 0.1 M glycine HCl (pH 2.5) and immediately neutralized with Tris-HCl (pH 8.8). In some experiments, antibodies eluted from MBP were used as a control.

Immunofluorescence—Cells on coverslips were washed with PBS containing 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$ and fixed with 3% formaldehyde in PBS for 15 min at room temperature. After washing with PBS and incubation with 50 mM NH_4Cl in PBS, cells were permeabilized by incubating with 0.1% Triton X-100 in PBS for 5 min. Cells were preincubated with a mixture (1:1) of PBS and Dulbecco's modified Eagle's medium with 10% fetal calf serum for 15 min and incubated sequentially with anti-reticulocalbin antibodies and goat anti-rabbit antibodies conjugated with fluorescein isothiocyanate (Jackson Laboratories) in the same solution for 30 min. For double staining, biotinylated ConA and rhodamine-labeled avidin (EY Laboratories) were included in the solutions containing anti-reticulocalbin antibodies or anti-rabbit antibodies, respectively. Stained cells were photographed with a Nikon microscope using Fujichrome 100 film. Images from the same slides were generated by a confocal scanning laser microscope (MRC500, Bio-Rad) on a video monitor and photographed using TriX film.

RESULTS

Isolation of Reticulocalbin cDNA Clones—Fig. 1 shows the scheme of isolated reticulocalbin cDNA clones. Clones O1, O9, and O32 were isolated from a cDNA library in λgt11 constructed from mouse teratocarcinoma OTT6050 cDNA by screening with antibodies against *D. biflorus* agglutinin-binding glycoproteins of the cells. These clones provide the 3′-sequence to the internal *Eco*RI site at nucleotide 476, but they did not extend further because *Eco*RI sites were not methylated during construction of the library. Clone O32–8 was obtained by screening a λgt11 library constructed with a cDNA, whose internal *Eco*RI sites were protected with *Eco*RI methylase, by plaque hybridization using the *Eco*RI-*Bam*HI fragment of clone O32 as a probe. To isolate clones M10 and M22, we used an oligonucleotide (30-mer) corresponding to nucleotides 625–655 of reticulocalbin cDNA as a primer for construction of another cDNA library. This library in λgt10 was screened by plaque hybridization using the 144-bp *Eco*RI-*Nde*I fragment of clone O32 as a probe. Northern hybridization of RNAs from teratocarcinoma OTT6050, embryonal

carcinoma cell line F9, and parietal endodermal cell line PYS-2 revealed that the cDNA clones hybridized a major mRNA species of 2.3 kilobase pairs and a minor species of 2.0 kilobase pairs (data not shown).

The composite nucleotide sequence of the cDNA clones and the deduced amino acid sequence of reticulocalbin are presented in Fig. 2. The nucleotide sequence contains 34 bp of the 5′-untranslated region and a 3′-untranslated region of 1016 bp. The coding region specifies a protein of 325 residues with an M_r of 38,112. The amino acid sequence of the first 20 amino acids of reticulocalbin has the typical features of a secretory leader peptide. There is a positively charged amino-terminal region and a hydrophobic central section followed by two small nonpolar residues with a single residue between them. This latter feature is thought to determine the cleavage point of the signal peptidase, which in this case could be between Ala-20 and Leu-21. Cleavage of this peptide would yield a mature protein of 305 residues with a predicted M_r of 36,200. The sequence of reticulocalbin does not contain a hydrophobic transmembrane segment, which suggests transfer of the entire protein into the lumen of the ER. There is one consensus site for *N*-linked glycosylation and a number of serine- or threonine-rich regions that could be *O*-glycosylated.

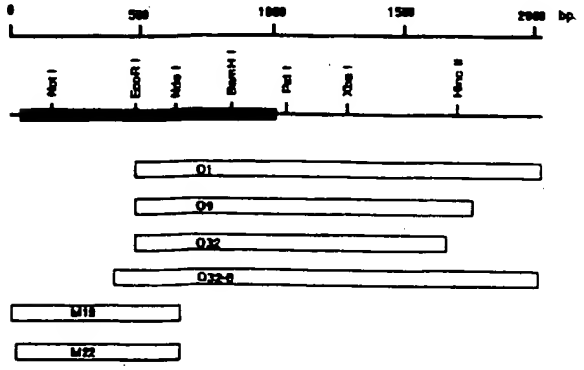

FIG. 1. **Restriction map and clones of reticulocalbin.** The scale indicates nucleotide positions beginning from the first base of clone M10. Beneath the scale, reticulocalbin mRNA is represented by a *thick black line* delineating the coding region. A partial restriction map is indicated. The *open boxes* correspond to the partial cDNA clones isolated from cDNA libraries.

Immunoblots of PYS-2 cells with antibodies against reticulocalbin revealed a band of 44 kDa and a faint band of 46 kDa (Fig. 3). The same bands were also detected in mouse fibroblast L cells (data not shown). The 46-kDa species binds to concanavalin A-Sepharose, whereas the 44-kDa species does not (Fig. 3). Neither of the two proteins bound to *R. communis* agglutinin-agarose, which recognizes terminal or sialylated Galβ1–4GlcNAc sequences (Baenzinger and Fiete, 1979). Therefore, the 46-kDa species may be produced by *N*-glycosylation (high-mannose type) of the 44-kDa species at residue 47. To confirm that the cDNA codes the 44-kDa protein, the cDNA was cloned into an expression vector (pCAGGSneo) and introduced into COS cells. Upon immunoblot analysis, COS cells transfected with the cDNA in a sense orientation showed a 44-kDa band and a faint 46-kDa band, whereas COS cells transfected with the cDNA in an antisense orientation gave faint bands in the region (Fig. 4). We consider that the faint bands (46 and 44 kDa) represent endogenous reticulocalbin in COS cells. Even though the 44-kDa band is present in COS cells transfected with antisense cDNA, the intensity of the band greatly increased in COS cells transfected with sense cDNA. Therefore, we conclude

FIG. 2. **Nucleotide sequence of reticulocalbin cDNA and predicted amino acid sequence of protein.** Nucleotides are numbered on the *right*. A signal sequence is denoted by the *underline*. A consensus *N*-glycosylation site is marked with a *filled triangle*. Residues corresponding to the calcium-binding loop of the EF-hands predicted from the homology of this family of calcium-binding proteins and the polyadenylation signals are *underlined*. In clones O9 and O32, which have a shorter 3′-flanking sequence, the poly(A) tails start at positions 1589 and 1744, respectively.

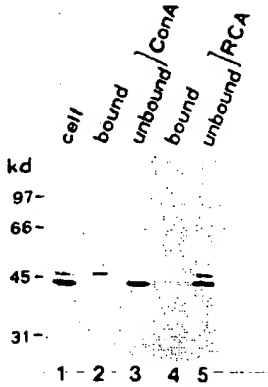

FIG. 3. **Immunoblot analysis of reticulocalbin.** PYS-2 cells were boiled in SDS-PAGE sample buffer (*lane 1*) or were lysed with Nonidet P-40 and fractionated on ConA-Sepharose (*lanes 2* and *3*) or *R. communis* agglutinin (*RCA*)-agarose (*lanes 4* and *5*). Bound materials specifically eluted with the respective haptenic sugars (*lanes 2* and *4*), and unbound materials (*lanes 3* and *5*) were immunoblotted with anti-reticulocalbin antibodies.

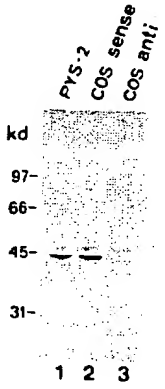

FIG. 4. **Immunoblots of reticulocalbin transiently expressed in COS cells.** PYS-2 (positive control) (*lane 1*) and COS cells transfected with reticulocalbin cDNA in an expression vector in a sense (*lane 2*) or antisense (*lane 3*) orientation were boiled in SDS-PAGE sample buffer and immunoblotted with anti-reticulocalbin antibodies. The antibodies recognized endogenous COS cell reticulocalbin in the immunoblots, but not in immunofluorescence and immunoprecipitation (compare with Figs. 8 and 9); probably the conformation-dependent epitopes detectable in these assays are species-specific.

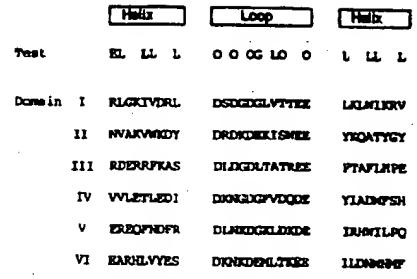

FIG. 5. **Calcium-binding domains of reticulocalbin.** The protein sequence (Fig. 2) was analyzed for homologies to the test sequence for a Ca^{2+}-binding EF-hand motif. The amino acid sequence is shown in one-letter-code. In the test sequence, *L* represents hydrophobic residues, and *O* represents oxygen-containing residues.

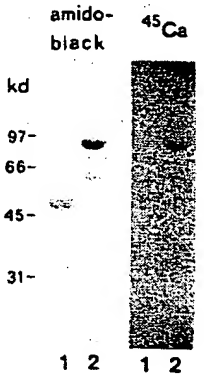

FIG. 6. **Calcium blots of recombinant reticulocalbin.** Maltose-binding protein (2 μg) (*lanes 1*) or a recombinant reticulocalbin of the maltose-binding protein fusion (2 μg) (*lanes 2*) purified on amylose resin was electrophoresed and transferred to a nitrocellulose membrane. After incubation with $^{45}Ca^{2+}$, the membrane was exposed to x-ray film. The same membrane was stained with Amido Black to detect proteins. The apparent molecular mass (88 kDa) of the fusion protein is in good agreement with the expected size (86 kDa).

that the cDNA clone has the entire sequence encoding the 44-kDa protein.

Reticulocalbin Is a Ca^{2+}-binding Protein with EF-hand Motifs—Analysis of the cDNA sequence shows that the predicted protein contains six repeats of ~30 amino acids (Fig. 5). Each repeat has the general feature of a high affinity Ca^{2+}-binding EF-hand domain according to Kretsinger's rule (Kretsinger, 1980). Comparing the amino acid sequence of these domains with those required for a perfect EF-hand Ca^{2+}-binding site, there is a varying degree of divergence. The 5 oxygen-containing residues important for the coordination of Ca^{2+} are present in all the predicted sites. The central glycine is conserved in sites I, IV, and V, whereas in sites II, III, and VI, it is replaced either by glutamic acid or leucine. The replacements appear not to be cloning artifacts because the independent cDNA clones have the same sequence in the region. Furthermore, the sequence of the corresponding region of genomic DNA clones also has the same sequence.[2] The secondary structure prediction (Chou and Fasman, 1978) shows that the replacement of glycine with glutamic acid in sites II and VI causes the formation of an α-helix at the sites instead of a loop structure. Therefore, sites II and VI of reticulocalbin probably no longer bind Ca^{2+}. Although the glycine is replaced by leucine, site III appears capable of forming the loop structure. The replacement of the normally conserved glycine with lysine has been reported in the EF-hands of the fibrinogen γ-chain (Dang *et al.*, 1985) and secreted protein acidic and rich in cysteine (BM-40, osteonectin) (Engel *et al.*, 1987). Therefore, site III seems to bind Ca^{2+}.

To test whether reticulocalbin actually binds Ca^{2+}, recombinant reticulocalbin was analyzed by $^{45}Ca^{2+}$ blotting. The cDNA coding mature reticulocalbin was cloned into the MBP fusion vector (pMAL-c), and the recombinant protein was expressed in *E. coli* as a fusion protein with MBP. After purification by affinity chromatography on a column containing amylose resin, the fusion protein was electrophoresed on a gel, transferred to a nitrocellulose membrane, and incubated with Ca^{2+}. MBP alone did not bind Ca^{2+}; however, the fusion protein did (Fig. 6).

Reticulocalbin Is a Luminal ER Protein—After homogenization of PYS-2 cells, reticulocalbin remained in the low speed (5000 × *g*) supernatant, but sedimented together with crude membranes during high speed (100,000 × *g*) centrifugation (Fig. 7*A*). It was solubilized from the membrane by Triton X-100 or by sonication in the absence of detergents (Fig. 7*A*). Reticulocalbin partitioned into the aqueous phase when cells

[2] M. Ozawa, unpublished results.

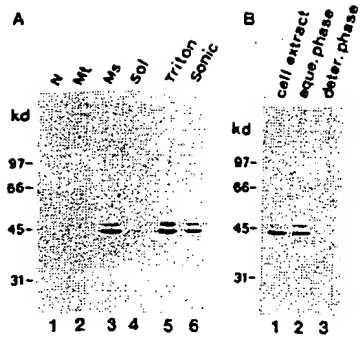

FIG. 7. **Reticulocalbin is a microsomal protein with a hydrophilic nature.** *A*, PYS-2 cells were homogenized and fractionated by differential centrifugation into nuclear (*N*; *lane 1*), mitochondrial (*Mt*; *lane 2*), microsomal (*Ms*; *lane 3*), and soluble (*Sol*; *lane 4*) fractions and immunoblotted. Reticulocalbin in the microsomal fraction was solubilized with Triton X-100 (*lane 5*) or released by sonication (*Sonic*; *lane 6*). *B*, cells were extracted with Triton X-114, and the extract (*lane 1*) was analyzed by phase separation. Reticulocalbin was found in the aqueous (*aque.*) phase (*lane 2*), and not in the detergent (*deter.*) phase (*lane 3*).

were extracted with Triton X-114 and phase-separated (Fig. 7*B*).

To localize reticulocalbin, PYS-2 cells were stained with anti-reticulocalbin antibodies by indirect immunofluorescence. When the cells were fixed with formaldehyde and permeabilized with Triton X-100, the intracellular ER regions (but not the cell surface) were stained (Fig. 8*A*). Fig. 8*A* shows that reticulocalbin is localized in the perinuclear system of membranes corresponding to that of the ER of PYS-2 cells. Since the ER seemed not to be well developed in PYS-2 cells, we stained transfected COS cells expressing reticulocalbin. Untransfected cells were not detectably stained with the antibody (data not shown). All cells expressing reticulocalbin exhibited prominent staining of the perinuclear region as well as a lattice of fine tubular structures (Fig. 8*C*), but there was no obvious staining of the Golgi apparatus. This pattern is typical of that obtained for proteins that are retained in the ER (Munro and Pelham, 1987). Furthermore, the structure that contained reticulocalbin also stained with concanavalin A (Fig. 8*D*), which predominantly stains ER structures (Tartakoff and Vassalli, 1983), confirming that they represented the ER. Because of the bright staining in the perinuclear region, however, it was difficult to get a clearly visible pattern of the tubule network. We overcame this issue using a confocal scanning laser microscope. Fig. 8*E* shows the distribution of reticulocalbin in the transfected COS cells. The image confirms the results obtained using conventional microscopy and allows detailed examination of the distribution of the protein. There is an intense reticular staining of the ER.

Reticulocalbin has a putative amino-terminal signal peptide, but no hydrophobic transmembrane segment, which is consistent with transfer of the entire protein into the lumen of the ER. To verify that the amino-terminal stretch of hydrophobic amino acids functions as a signal sequence, we carried out the experiments described below. The rationale is as follows. Luminal ER proteins in animal cells are prevented from being secreted by a sorting system that recognizes the carboxyl-terminal sequence KDEL (Munro and Pelham, 1987). Instead of KDEL at the carboxyl terminus, reticulocalbin has the closely related sequence HDEL. Although the HDEL sequence has been reported to be inefficient as an ER retention signal in animal cells because a lysozyme fusion protein with the HDEL sequence at the carboxyl terminus was efficiently secreted into the medium (Pelham *et al.*, 1988),

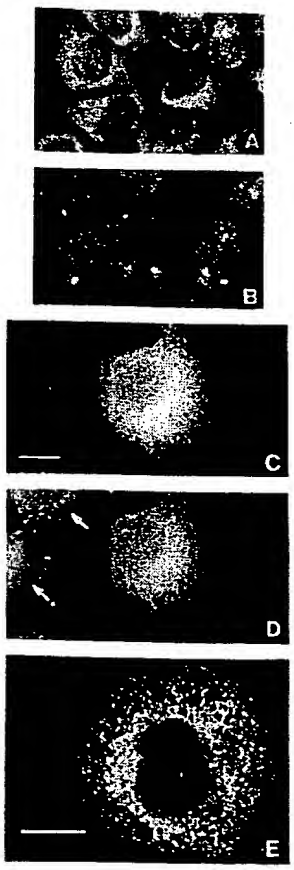

FIG. 8. **Localization of reticulocalbin by immunofluorescent staining.** PYS-2 cells (*A* and *B*) or COS cells transfected with reticulocalbin cDNA (*C–E*) were stained with anti-reticulocalbin antibodies (*A*, *C*, and *E*) or control antibodies (*B*). In *C* and *D*, cells were double-labeled with anti-reticulocalbin antibody and biotinylated ConA followed by fluorescein-labeled goat anti-rabbit antibody and rhodamine-labeled avidin, and distribution of reticulocalbin (*C*) and ConA (*D*) was visualized. *Arrows* in *D* shows cells negative for reticulocalbin staining (*C*). *A–D*, cells were photographed using an immunofluorescence microscope at the same magnification. *Bar*, 20 μm. *E*, an image generated by an MRC500 confocal scanning laser microscope on a video monitor. *Bar*, 25 μm. The microscope was focused on the lower part of the cell.

recent experiments on liver carboxylesterases have demonstrated that the sequence is functional at least in this case (Robbi and Beaufay, 1991). Therefore, the removal of the carboxyl-terminal HDEL sequence could allow the mutant protein to escape from the retrieval machinery and to be secreted into the medium if the amino-terminal sequence is the signal sequence. For this, a mutant protein specifically lacking the HDEL sequence was constructed. The synthesis and secretion of the wild-type and mutant reticulocalbins were evaluated by transient expression in COS cells (Fig. 9). After labeling with [^{35}S]methionine for 30 min and a chase (3 h) with excess unlabeled methionine, proteins were immunoprecipitated from the cell lysates and from media and then analyzed by SDS-PAGE. Under these conditions, wild-type reticulocalbin was secreted very slowly from the COS cells. Less than 10% of the pulse-labeled protein was found in the medium. Reticulocalbin lacking the HDEL sequence, however, was secreted rapidly from the COS cells (Fig. 9). Densitometry of the fluorographs showed that ~80% of the mutant protein was recovered from the medium. No reticulocalbin was secreted from PYS-2 cells under the same conditions (data not shown). These results show that the amino-terminal

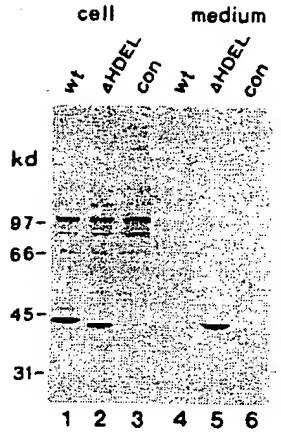

FIG. 9. **Immunoprecipitation of wild-type reticulocalbin and mutant reticulocalbin lacking carboxyl-terminal HDEL peptide.** COS cells transfected with wild-type (*wt*) (*lanes 1* and *4*) or mutant (*ΔHDEL*) (*lanes 2* and *5*) reticulocalbin cDNA in an expression vector or the vector without the cDNA (control (*con*)) (*lanes 3* and *6*) were labeled with [^{35}S]methionine for 30 min. After a chase for 2 h with excess unlabeled methionine, the cells and media were collected. The cell lysates (*lanes 1–3*) and media (*lanes 4–6*) were incubated with anti-reticulocalbin antibodies, and immunocomplexes were analyzed by SDS-PAGE.

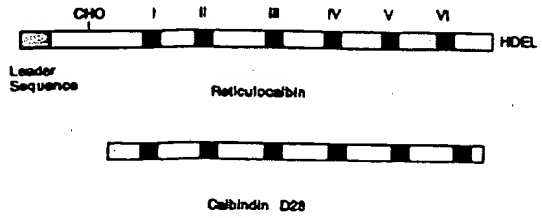

FIG. 10. **Domain structure of reticulocalbin and calbindin D28.** The *filled boxes* represent the sequences corresponding to the loop of the EF-hand structure. The consensus *N*-glycosylation site is marked by *CHO*.

hydrophobic amino acids of reticulocalbin function as a signal for the transfer of the entire protein into the lumen of the ER.

DISCUSSION

We identified and characterized a novel ER resident Ca^{2+}-binding protein called reticulocalbin by cDNA cloning, sequence analysis, and biochemical as well as cell biological studies. The major structural features of reticulocalbin, as deduced from the cDNA sequence, are outlined in Fig. 10. The protein consists of 325 amino acids with a single hydrophobic sequence at the amino terminus that constitutes a leader sequence. At the amino-terminal region of the mature protein, there is a potential *N*-glycosylation site, which was indeed partially glycosylated. The carboxyl terminus contains a version of the KDEL ER retention signal, HDEL. The rest of the protein consists of the six domains of the EF-hand motif of high affinity Ca^{2+}-binding proteins.

The most interesting feature of the sequence of reticulocalbin is the presence of the domains of the EF-hand motif. Therefore, reticulocalbin can be classified into the EF-hand calcium-binding protein superfamily, which includes calmodulin, troponin C, and myosin light chain. All members of this diverse protein family share multiple conserved sequence domains based on a distinct helix-loop-helix structure, the EF-hand (Kretsinger, 1980). The loop constitutes the Ca^{2+}-binding site. The proteins of the superfamily identified to date contain from two to eight EF-hands or variants thereof. In some of them, the EF-hands have been duplicated r lost; and in others, the calcium binding properties have been altered or lost entirely (Heizman and Hunziker, 1991).

Although the reticulocalbin sequence has no significant homology to any other proteins except for the EF-hand motifs, the overall structure of reticulocalbin is similar to that of calbindin D28 and calretinin (Rogers, 1989) in that both proteins have six EF-hand motif domains. Calbindin D28 and calretinin have been found at high concentrations in the central and peripheral nervous systems of many species, but their function is presently unknown. Calbindin D28 binds only four Ca^{2+} atoms/mol of protein, and the second and sixth domains may have lost their Ca^{2+} binding capability because some oxygen-containing amino acids in the loop are missing (Hunziker, 1986). Similarly, the second and sixth domains of reticulocalbin seem to have lost their Ca^{2+} binding ability. Reticulocalbin, however, has a long amino-terminal extension as well as a short carboxyl-terminal extension. The latter has an HDEL sequence that could serve as part of the retention signal for the protein in the ER. The amino-terminal extension is composed of a leader sequence, which directs the protein to be translocated into the lumen of the ER, and the amino-terminal region of the mature protein of ~50 amino acids, where a single *N*-glycosylation site resides.

The evidence presented here demonstrates that reticulocalbin is a luminal Ca^{2+}-binding protein residing in the ER. The sequence of the cDNA clone showed that it has a secretory leader peptide that is not present on the mature protein. This leader sequence is functional in that it causes reticulocalbin to enter the ER, as shown by its glycosylation, and to be secreted into the medium since a mutant reticulocalbin lacking the carboxyl-terminal HDEL sequence was secreted in COS cells. Mature reticulocalbin does not contain a hydrophobic transmembrane sequence, and there is no evidence for its secretion from the cells; it thus appears likely that it accumulates as a soluble protein within the intracellular membrane system. Consistent with this is the finding that reticulocalbin cosediments with crude membranes (microsomes) and can be released from the membranes with detergents or by sonication. Upon phase separation in Triton X-114, reticulocalbin partitioned into the aqueous phase. Furthermore, anti-reticulocalbin antibody stained endogenous reticulocalbin in PYS-2 cells as well as transiently expressed reticulocalbin in COS cells with an ER-like pattern.

The carboxyl-terminal HDEL tetrapeptide, a variant of the KDEL ER retention signal, seems to be part of a signal that prevents it from being secreted from the cell since the mutant reticulocalbin lacking HDEL is secreted. After identifying the carboxyl-terminal KDEL sequence as an ER retention signal, a number of variants of this sequence have been reported (Pelham, 1990), including sequences KEEL for the protein ERp72 (Mazzarella *et al.*, 1990) and RDEL in a 55-kDa thyroid hormone-binding protein (Fliegel *et al.*, 1990). The retrieval system in the yeast *Saccharomyces cerevisiae* recognizes the carboxyl-terminal HDEL sequence of ER resident proteins (Pelham *et al.*, 1988). The HDEL sequence, however, is reportedly inefficient as an ER retention signal in animal cells because addition of the sequence to a lysozyme fusion protein did not cause its retention in the ER when the protein was expressed in COS cells (Pelham *et al.*, 1988). Despite this observation, HDEL can be used as the ER retention signal in some ER resident proteins such as reticulocalbin because the efficiency of retention can vary, depending on the proteins to be analyzed, as has been reported (Zagouras and Rose, 1989). Recently, HDEL in carboxylesterase has been shown to be

functional in animal cells (Robbi and Beaufay, 1991). The other possibility, that the removal of HDEL caused altered protein folding, which in turn affected the interaction of reticulocalbin with other ER resident proteins, however, is not formally excluded.

Although our studies revealed valuable information about the structure and localization of reticulocalbin, the function of this protein remains unknown. Its localization in the lumen of the ER and its expression in different types of cells suggest a role in protein synthesis, modification, and intracellular transport. We speculate that reticulocalbin functions in the regulation of Ca^{2+}-dependent activities in the lumen of the ER or post-ER compartment. One intriguing possibility is that reticulocalbin is involved in the retention mechanism of KDEL-terminated proteins in the ER, in which Ca^{2+} may be involved (Booth and Koch, 1989; Kelly, 1990). Reticulocalbin may associate with an as yet unidentified protein and regulate its activity by binding Ca^{2+}. That reticulocalbin may perform multiple functions like calmodulin should be considered.

Finally, we believe that the name reticulocalbin is appropriate for the protein because it reflects its intracellular localization in the lumen of the ER (reticuloplasm), established Ca^{2+} binding properties, and the six calbindin D28-like domains of the EF-hand motifs.

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SeqServer
biology in silico

ClustalW Results

Sequences | Help

Retrieval | BLAST2 | FASTA | ClustalW | GCG Assembly | Phrap | Translation

- [ ] 1601793CD1
- [ ] g220582

CLUSTAL W (1.7) Multiple Sequence Alignments

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Sequence format is Pearson
Sequence 1: 1601793CD1      315 aa
Sequence 2: g220582         325 aa
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  58
Start of Multiple Alignment
There are 1 groups
Aligning...
Group 1: Sequences:   2      Score:3364
Alignment Score 1226
CLUSTAL-Alignment file created  [baax0ayYk.aln]
CLUSTAL W (1.7) multiple sequence alignment


1601793CD1      -MDLRQFLMCLSLCTAFALS---KPTEKKDRVHH-EPQLSDKVHNDAQSFDYDHDAFLGA
g220582         MARGGRLGLALGLLLALVLALRAKPTVRKERVVRPDSELGERPPEDNQSFQYDHEAFLGK
                     :: :.*.*  *:.*:   *** :*:** : :.:*.::  :* ***:***:****

1601793CD1      EEAKTFDQLTPEESKERLGKIVSKIDGDKDGFVTVDELKDWIKFAQKRWIYEDVERQWKG
g220582         EDSKTFDQLSPDESKERLGKIVDRIDSDGDGLVTTEELKLWIKRVQKRYIYDNVAKVWKD
                *:::******:*:**********.:**.* **:**.:*** *** .***:**::* : **.

1601793CD1      HDLNEDGLVSWEEYKNATYGYVLDDP----DPDDGFNYKQMMVRDERRFKMADKDGDLIA
g220582         YDRDKDEKISWEEYKQATYGYYLGNPAEFHDSSDHHTFKKMLPRDERRFKASDLDGDLTA
                :* ::*  :******:***** *.:*    *..* ..:*:*: ******* :* **** *

1601793CD1      TKEEFTAFLHPEEYDYMKDIVVQETMEDIDKNADGFIDLEEYIGDMYSHDGNTDEPEWVK
g220582         TREEFTAFLHPEEFEHMKEIVVLETLEDIDKNGDGFVDQDEYIADMFSHEDNGPEPDWVL
                *:***********:::**:*** **:******.***:* :***.**:**:.*  **:**

1601793CD1      TEREQFVEFRDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIV
g220582         SEREQFNDFRDLNKDGKLDKDEIRHWILPQDYDHAQAEARHLVYESDKNKDEMLTKEEIL
                :***** :*** *:***:**:* :.****.*****:***********:***  ******:

1601793CD1      DKYDLFVGSQATDFGEALVR-HDEF
g220582         DNWNMFVGSQATNYGEDLTKNHDEL
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Sequence Revision History

Search for Genes

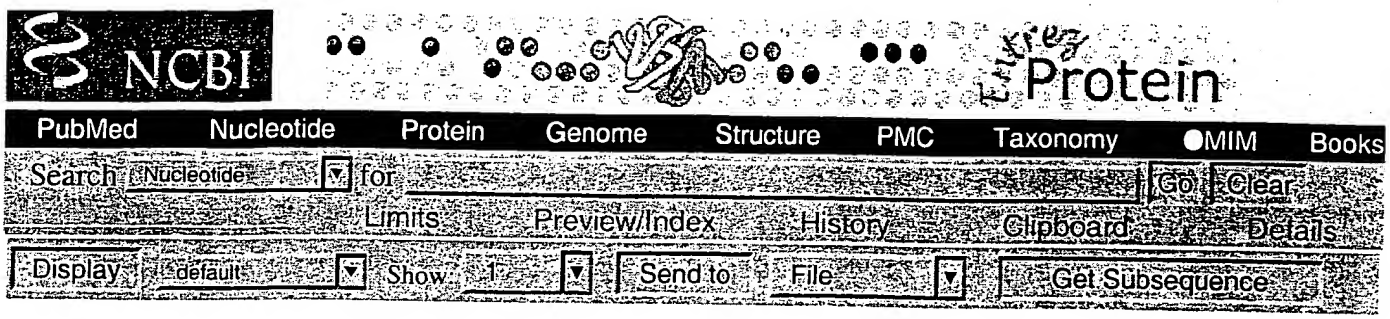
Entrez Nucleotide

Revision history for "D13003"

GI	Version	Update Date	Status
220581	1	Jul 25 2002 14:17	Live
220581	1	Sep 8 1999 19:10	Dead
220581	1	Mar 17 1999 21:53	Dead
220581	1	Jun 5 1997 14:48	Dead
220581	1	Apr 29 1993 10:48	Dead

Accession D13003 was first seen at NCBI on Apr 29 1993 10:48

Related resources



1: BAA02366. reticulocalbin [M...[gi:220582] BLink, Domains, Links

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DEFINITION  reticulocalbin [Mus musculus].
ACCESSION   BAA02366
VERSION     BAA02366.1  GI:220582
DBSOURCE    locus MUSRCAL accession D13003.1
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (residues 1 to 325)
  AUTHORS   Ozawa,M. and Muramatsu,T.
  TITLE     Reticulocalbin, a novel endoplasmic reticulum resident
            Ca(2+)-binding protein with multiple EF-hand motifs and a
            carboxyl-terminal HDEL sequence
  JOURNAL   J. Biol. Chem. 268 (1), 699-705 (1993)
  MEDLINE   93107083
   PUBMED   8416973
REFERENCE   2  (residues 1 to 325)
  AUTHORS   Ozawa,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-AUG-1992) Masayuki Ozawa, Faculty of Medicine,
            Kagoshima University, Department of Biochemistry; 8-35-1
            Sakuragaoka, Kagoshima, Kagoshima 890, Japan (Tel:0992-75-5246,
            Fax:0992-64-5618)
FEATURES             Location/Qualifiers
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                     /clone="O1 and M10"
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     sig_peptide     1..20
     mat_peptide     21..325
                     /product="unnamed"
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                     /coded_by="D13003.1:35..1012"
ORIGIN
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       61 edsktfdqls pdeskerlgk ivdridsdgd glvtteelkl wikrvqkryi ydnvakvwkd
      121 ydrdkdekis weeykqatyg yylgnpaefh dssdhhtfkk mlprderrfk asdldgdlta
      181 treeftaflh peefehmkei vvletledid kngdgfvdqd eyiadmfshe dngpepdwvl
      241 sereqfndfr dlnkdgkldk deirhwilpq dydhaqaear hlvyesdknk demltkeeil
      301 dnwnmfvgsq atnygedltk nhdel
//
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Qy    181 HPEEYDYMKDIVVQETMEDIDKNADGFIDLEEYIGDMYSHDGNTDEPEWVKTEREQFVEF 240
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Db    181 HPEEYDYMKDIVVQETMEDIDKNADGFIDLEEYIGDMYSHDGNTDEPEWVKTEREQFVEF 240

Qy    241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGS 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db    241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGS 300

Qy    301 QATDFGEALVRHDEF 315
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Db    301 QATDFGEALVRHDEF 315


RESULT 2
O35783
ID   O35783      PRELIMINARY;      PRT;   315 AA.
AC   O35783;
DT   01-JAN-1998 (TrEMBLrel. 05, Created)
DT   01-JAN-1998 (TrEMBLrel. 05, Last sequence update)
DT   01-MAR-2002 (TrEMBLrel. 20, Last annotation update)
DE   CBP-50 protein.
OS   Rattus norvegicus (Rat).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Rattus.
OX   NCBI_TaxID=10116;
RN   [1]
RP   SEQUENCE FROM N.A.
RC   STRAIN=SPRAGUE-DAWLEY; TISSUE=BRAIN;
RA   Hseu M.J.;
RL   Submitted (OCT-1997) to the EMBL/GenBank/DDBJ databases.
RN   [2]
RP   SEQUENCE FROM N.A.
RC   STRAIN=SPRAGUE-DAWLEY; TISSUE=BRAIN;
RX   MEDLINE=93107083; PubMed=8416973;
RA   Ozawa M., Muramatsu T.;
RT   "Reticulocalbin: a novel endoplasmic reticulum resident calcium-
RT   binding protein with multiple EF-hand motifs and a caboxyl-terminal
RT   HDEL sequence.";
RL   J. Biol. Chem. 268:699-705(1993).
DR   EMBL; AJ001929; CAA05100.1; -.
DR   InterPro; IPR002048; EF-hand.
DR   Pfam; PF00036; efhand; 6.
DR   SMART; SM00054; EFh; 2.
DR   PROSITE; PS00018; EF_HAND; UNKNOWN_4.
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  Best Local Similarity   88.6%;  Pred. No. 8.3e-89;
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Qy    241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGS 300
          ||||||||||||||||||||||||||||||||||:||||||||||||||||||||||||||
Db    241 RDKNRDGKMDKEETKDWILPSDYDHAEAEARHLVYESDQDKDGKLTKEEIVDKYDLFVGS 300

Qy    301 QATDFGEALVRHDEF 315
          |||||||||||||||
Db    301 QATDFGEALVRHDEF 315


RESULT 3
Q9NR43
ID   Q9NR43      PRELIMINARY;      PRT;   296 AA.
AC   Q9NR43;
DT   01-OCT-2000 (TrEMBLrel. 15, Created)
DT   01-OCT-2000 (TrEMBLrel. 15, Last sequence update)
DT   01-JUN-2002 (TrEMBLrel. 21, Last annotation update)
DE   Crocalbin-like protein (Fragment).
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
OX   NCBI_TaxID=9606;
RN   [1]
RP   SEQUENCE FROM N.A.
RC   TISSUE=BRAIN;
RX   MEDLINE=99192326; PubMed=10094503;
RA   Hseu M.J., Yen C.H., Tzeng M.C.;
RT   "Crocalbin: a new calcium-binding protein that is also a binding
RT   protein for crotoxin, a neurotoxic phospholipase A2.";
RL   FEBS Lett. 445:440-444(1999).
RN   [2]
RP   SEQUENCE FROM N.A.
RC   TISSUE=BRAIN;
RA   Hseu M.J., Tzeng M.C.;
RL   Submitted (APR-2000) to the EMBL/GenBank/DDBJ databases.
DR   EMBL; AF257659; AAF76141.1; -.
DR   InterPro; IPR002048; EF-hand.
DR   Pfam; PF00036; efhand; 6.
DR   SMART; SM00054; EFh; 3.
DR   PROSITE; PS00018; EF_HAND; UNKNOWN_4.
FT   NON_TER       1      1
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  Query Match             87.7%;  Score 1491;  DB 4;  Length 296;
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Qy     80 IDGDKDGFVTVDELKDWIKFAQKRWIYEDVERQWKGHDLNEDGLVSWEEYKNATYGYVLD 139
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Qy    200 IDKNADGFIDLEEYIGDMYSHDGNTDEPEWVKTEREQFVEFRDKNRDGKMDKEETKDWIL 259
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Qy    260 PSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGSQATDFGEALVRHDEF 315
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db    241 PSDYDHAEAEARHLVYESDQNKDGKLTKEEIVDKYDLFVGSQATDFGEALVRHDEF 296


RESULT 4
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ID   Q9MZ46      PRELIMINARY;      PRT;   296 AA.
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DT   01-OCT-2000 (TrEMBLrel. 15, Created)
DT   01-OCT-2000 (TrEMBLrel. 15, Last sequence update)
DT   01-MAR-2002 (TrEMBLrel. 20, Last annotation update)
DE   Crocalbin-like protein (Fragment).
OS   Sus scrofa (Pig).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Cetartiodactyla; Suina; Suidae; Sus.
OX   NCBI_TaxID=9823;
RN   [1]
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